TITLE OF THE INVENTION

SPECIES-SPECIFIC, GENUS-SPECIFIC AND UNIVERSAL DNA PROBES AND AMPLIFICATION PRIMERS TO RAPIDLY DETECT AND IDENTIFY COMMON BACTERIAL AND FUNGAL PATHOGENS AND ASSOCIATED ANTIBIOTIC RESISTANCE GENES FROM CLINICAL SPECIMENS FOR DIAGNOSIS IN MICROBIOLOGY LABORATORIES

BACKGROUND OF THE INVENTION

Classical methods for the identification and susceptibility testing of bacteria

Bacteria are classically identified by their ability to utilize different substrates as a source of carbon and nitrogen through the use of biochemical tests such as the API20E™ system (bioMérieux). For susceptibility testing, clinical microbiology laboratories use methods including disk diffusion, agar dilution and broth microdilution. Although identifications based on biochemical tests and antibacterial susceptibility tests are cost-effective, at least two days are required to obtain preliminary results due to the necessity of two successive overnight incubations to identify the bacteria from clinical specimens as well as to determine their susceptibility to antimicrobial agents. There are some commercially available automated systems (i.e. the MicroScan system from Dade Diagnostics Corp. and the Vitek system from bioMérieux) which use sophisticated and expensive apparatus for faster microbial identification and susceptibility testing (Stager and Davis, 1992, Clin. Microbiol. Rev. **5**:302-327). These systems require shorter incubation periods, thereby allowing most bacterial identifications and susceptibility testing to be performed in less than 6 hours. Nevertheless, these faster systems always require the primary isolation of the bacteria as a pure culture, a process which takes at least 18 hours for a pure culture or 2 days for a mixed culture. The fastest identification system, the autoSCAN-Walk-Away™ system (Dade Diagnostics Corp.) identifies both gram-negative and gram-positive bacterial species from standardized inoculum in as little as 2 hours and gives susceptibility patterns to most antibiotics in 5.5 hours. However, this system has a particularly high percentage (i.e. 3.3 to 40.5%) of non-conclusive identifications with bacterial species other than *Enterobacteriaceae* (Croizé J., 1995, Lett. Infectiol. **10**:109-113; York *et al.*, 1992, J. Clin. Microbiol. **30**:2903-2910). For *Enterobacteriaceae*, the percentage of non-conclusive identifications was 2.7 to 11.4%.

A wide variety of bacteria and fungi are routinely isolated and identified from clinical specimens in microbiology laboratories. Tables 1 and 2 give the incidence for the most commonly isolated bacterial and fungal pathogens from various types of clinical specimens. These pathogens are the most frequently associated with nosocomial and community-acquired human infections and are therefore considered the most clinically important.

Clinical specimens tested in clinical microbiology laboratories

Most clinical specimens received in clinical microbiology laboratories are urine and blood samples. At the microbiology laboratory of the Centre Hospitalier de l'Université Laval (CHUL), urine and blood account for approximately 55% and 30% of the specimens received, respectively (Table 3). The remaining 15% of clinical specimens comprise various biological fluids including sputum, pus, cerebrospinal fluid, synovial fluid, and others (Table 3). Infections of the urinary tract, the respiratory tract and the bloodstream are usually of bacterial etiology and require antimicrobial therapy. In fact, all clinical samples received in the clinical microbiology laboratory are tested routinely for the identification of bacteria and susceptibility testing.

Conventional pathogen identification from clinical specimens

Urine specimens

The search for pathogens in urine specimens is so preponderant in the routine microbiology laboratory that a myriad of tests have been developed. However, the gold standard remains the classical semi-quantitative plate culture method in which 1 µL of urine is streaked on plates and incubated for 18-24 hours. Colonies are then counted to determine the total number of colony forming units (CFU) per liter of urine. A bacterial urinary tract infection (UTI) is normally associated with a bacterial count of 10^7 CFU/L or more in urine. However, infections with less than 10^7 CFU/L in urine are possible, particularly in patients with a high incidence of diseases or those catheterized (Stark and Maki, 1984, N. Engl. J. Med. **311**:560-564). Importantly, approximately 80% of urine specimens tested in clinical microbiology laboratories are considered negative (i.e. bacterial count of less than 10^7 CFU/L; Table 3). Urine specimens found positive by culture are further characterized using standard biochemical tests to identify the bacterial pathogen and are also tested for susceptibility to antibiotics. The biochemical and susceptibility testing normally require 18-24 hours of incubation.

Accurate and rapid urine screening methods for bacterial pathogens would allow a faster identification of negative specimens and a more efficient treatment and care management of patients. Several rapid identification methods (Uriscreen™, UTIscreen™, Flash Track™ DNA probes and others) have been compared to slower standard biochemical methods, which are based on culture of the bacterial pathogens. Although much faster, these rapid tests showed low sensitivities and poor specificities as well as a high number of false negative and false positive results (Koening *et al.*, 1992, J. Clin. Microbiol. **30**:342-345; Pezzlo *et al.*, 1992, J. Clin. Microbiol. **30**:640-684).

Blood specimens

The blood specimens received in the microbiology laboratory are always submitted for culture. Blood culture systems may be manual, semi-automated or completely automated. The BACTEC system (from Becton Dickinson) and the

BacTAlert system (from Organon Teknika Corporation) are the two most widely used automated blood culture systems. These systems incubate blood culture bottles under optimal conditions for bacterial growth. Bacterial growth is monitored continuously to detect early positives by using highly sensitive bacterial growth detectors. Once growth is detected, a Gram stain is performed directly from the blood culture and then used to inoculate nutrient agar plates. Subsequently, bacterial identification and susceptibility testing are carried out from isolated bacterial colonies with automated systems as described previously. The bottles are normally reported as negative if no growth is detected after an incubation of 6 to 7 days. Normally, the vast majority of blood cultures are reported negative. For example, the percentage of negative blood cultures at the microbiology laboratory of the CHUL for the period February 1994-January 1995 was 93.1% (Table 3).

Other clinical samples

Upon receipt by the clinical microbiology laboratory, all body fluids other than blood and urine that are from normally sterile sites (i.e. cerebrospinal, synovial, pleural, pericardial and others) are processed for direct microscopic examination and subsequent culture. Again, most clinical samples are negative for culture (Table 3).

Regarding clinical specimens which are not from sterile sites such as sputum or stool specimens, the laboratory diagnosis by culture is more problematic because of the contamination by the normal flora. The bacterial pathogens potentially associated with the infection are purified from the contaminants and then identified as described previously. Of course, the universal detection of bacteria would not be useful for the diagnosis of bacterial infections at these non sterile sites. On the other hand, DNA-based assays for species or genus detection and identification as well as for the detection of antibiotic resistance genes from these specimens would be very useful and would offer several advantages over classical identification and susceptibility testing methods.

DNA-based assays with any clinical specimens

There is an obvious need for rapid and accurate diagnostic tests for bacterial detection and identification directly from clinical specimens. DNA-based technologies are rapid and accurate and offer a great potential to improve the diagnosis of infectious diseases (Persing *et al.*, 1993, Diagnostic Molecular Microbiology: Principles and Applications, American Society for Microbiology, Washington, D.C.). The DNA probes and amplification primers which are objects of the present invention are applicable for bacterial or fungal detection and identification directly from any clinical specimens such as blood cultures, blood, urine, sputum, cerebrospinal fluid, pus and other type of specimens (Table 3). The DNA-based tests proposed in this invention are superior in terms of both rapidity and accuracy to standard biochemical methods currently used for routine diagnosis from any clinical specimens in microbiology laboratories. Since

these tests are performed in around only one hour, they provide the clinicians with new diagnostic tools which should contribute to increase the efficiency of therapies with antimicrobial agents. Clinical specimens from organisms other than humans (e.g. other primates, birds, plants, mammals, farm animals, livestock and others) may also be tested with these assays.

A high percentage of culture negative specimens

Among all the clinical specimens received for routine diagnosis, approximately 80% of urine specimens and even more (around 95%) for other types of clinical specimens are negative for the presence of bacterial pathogens (Table 3). It would also be desirable, in addition to identify bacteria at the species or genus level in a given specimen, to screen out the high proportion of negative clinical specimens with a test detecting the presence of any bacterium (i.e. universal bacterial detection). Such a screening test may be based on the DNA amplification by PCR of a highly conserved genetic target found in all bacteria. Specimens negative for bacteria would not be amplified by this assay. On the other hand, those that are positive for bacteria would give a positive amplification signal with this assay.

Towards the development of rapid DNA-based diagnostic tests

A rapid diagnostic test should have a significant impact on the management of infections. DNA probe and DNA amplification technologies offer several advantages over conventional methods for the identification of pathogens and antibiotic resistance genes from clinical samples (Persing *et al.*, 1993, Diagnostic Molecular Microbiology: Principles and Applications, American Society for Microbiology, Washington, D.C.; Ehrlich and Greenberg, 1994, PCR-based Diagnostics in Infectious Disease, Blackwell Scientific Publications, Boston, MA). There is no need for culture of the bacterial pathogens, hence the organisms can be detected directly from clinical samples, thereby reducing the time associated with the isolation and identification of pathogens. Furthermore, DNA-based assays are more accurate for bacterial identification than currently used phenotypic identification systems which are based on biochemical tests. Commercially available DNA-based technologies are currently used in clinical microbiology laboratories, mainly for the detection and identification of fastidious bacterial pathogens such as *Mycobacterium tuberculosis*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae* as well as for the detection of a variety of viruses (Podzorski and Persing, Molecular detection and identification of microorganisms, *In* : P. Murray *et al.*, 1995, Manual of Clinical Microbiology, ASM press, Washington D.C.). There are also other commercially available DNA-based assays which are used for culture confirmation assays.

Others have developed DNA-based tests for the detection and identification of bacterial pathogens which are objects of the present invention: *Staphylococcus* spp. (US patent No. US 5 437 978), *Neisseria* spp. (US patent No. US 5 162 199 and European patent publication No. EP 0 337 896 131) and *Listeria monocytogenes* (US patents Nos 5 389 513 and 5 089 386). However, the diagnostic tests described in

these patents are based either on rRNA genes or on genetic targets different from those described in the present invention.

Although there are diagnostic kits or methods already used in clinical microbiology laboratories, there is still a need for an advantageous alternative to the conventional culture identification methods in order to improve the accuracy and the speed of the diagnosis of commonly encountered bacterial infections. Besides being much faster, DNA-based diagnostic tests are more accurate than standard biochemical tests presently used for diagnosis because the bacterial genotype (e.g. DNA level) is more stable than the bacterial phenotype (e.g. metabolic level).

Knowledge of the genomic sequences of bacterial and fungal species continuously increases as testified by the number of sequences available from databases. From the sequences readily available from databases, there is no indication therefrom as to their potential for diagnostic purposes. For determining good candidates for diagnostic purposes, one could select sequences for DNA-based assays for (i) the species-specific detection and identification of commonly encountered bacterial or fungal pathogens, (ii) the genus-specific detection and identification of commonly encountered bacterial or fungal pathogens, (iii) the universal detection of bacterial or fungal pathogens and/or (iv) the specific detection and identification of antibiotic resistance genes. All of the above types of DNA-based assays may be performed directly from any type of clinical specimens or from a microbial culture.

In WO 96/08502 patent publication, we described DNA sequences suitable for (i) the species-specific detection and identification of 12 clinically important bacterial pathogens, (ii) the universal detection of bacteria, and (iii) the detection of 17 antibiotic resistance genes. This co-pending application described proprietary DNA sequences and DNA sequences selected from databases (in both cases, fragments of at least 100 base pairs), as well as oligonucleotide probes and amplification primers derived from these sequences. All the nucleic acid sequences described in this patent application enter the composition of diagnostic kits and methods capable of a) detecting the presence of bacteria, b) detecting specifically the presence of 12 bacterial species and 17 antibiotic resistance genes. However, these methods and kits need to be improved, since the ideal kit and method should be capable of diagnosing close to 100% of microbial pathogens and antibiotic resistance genes. For example, infections caused by *Enterococcus faecium* have become a clinical problem because of its resistance to many antibiotics. Both the detection of these bacteria and the evaluation of their resistance profiles are desirable. It is worthwhile noting that the French patent publication FR-A-2,699,539 discloses the sequence of vancomycin B gene, which gene may be derived from *Enterococcus faecium* strains resistant to this antibiotic. Besides that, novel DNA sequences (probes and primers) capable of recognizing the same and other microbial pathogens or the same and additional antibiotic resistance genes are also desirable to aim at detecting more target genes and complement our

earlier patent application.

STATEMENT OF THE INVENTION

It is an object of the present invention to provide a specific, ubiquitous and sensitive method using probes and/or amplification primers for determining the presence and/or amount of nucleic acids:

- from specific microbial species or genera selected from the group consisting of *Streptococcus* species, *Streptococcus agalactiae*, *Staphylococcus* species, *Staphylococcus saprophyticus*, *Enterococcus* species, *Enterococcus faecium*, *Neisseria* species, *Neisseria meningitidis*, *Listeria monocytogenes*, *Candida* species and *Candida albicans*

- from an antibiotic resistance gene selected from the group consisting of bla_{tem}, bla_{rob}, bla_{shv}, bla_{oxa}, *blaZ, aadB, aacC1, aacC2, aacC3, aacA4, aac6'-IIa, ermA, ermB, ermC, mecA, vanA, vanB, vanC, satA, aac(6')-aph(2"), aad(6'), vat, vga, msrA, sul* and *int*, and optionally,

- from any bacterial species

in any sample suspected of containing said nucleic acids,

wherein each of said nucleic acids or a variant or part thereof comprises a selected target region hybridizable with said probe or primers;

said method comprising the steps of contacting said sample with said probes or primers and detecting the presence and/or amount of hybridized probes or amplified products as an indication of the presence and/or amount of said any bacterial species, specific microbial species or genus and antibiotic resistance gene.

In a specific embodiment, a similar method directed to each specific microbial species or genus detection and identification, antibiotic resistance genes detection, and universal bacterial detection, separately, is provided.

In a more specific embodiment, the method makes use of DNA fragments (proprietary fragments and fragments obtained from databases), selected for their capacity to sensitively, specifically and ubiquitously detect the targeted bacterial or fungal nucleic acids.

In a particularly preferred embodiment, oligonucleotides of at least 12 nucleotides in length have been derived from the longer DNA fragments, and are used in the present method as probes or amplification primers.

The proprietary oligonucleotides (probes and primers) are also another object of the invention.

Diagnostic kits comprising probes or amplification primers for the detection of

a microbial species or genus selected from the group consisting of *Streptococcus* species, *Streptococcus agalactiae*, *Staphylococcus* species, *Staphylococcus saprophyticus*, *Enterococcus* species, *Enterococcus faecium*, *Neisseria* species, *Neisseria meningitidis*, *Listeria monocytogenes, Candida* species and *Candida albicans* are also objects of the present invention.

Diagnostic kits further comprising probes or amplification primers for the detection of an antibiotic resistance gene selected from the group consisting of bla_{tem}, bla_{rob}, bla_{shv}, bla_{oxa}, *blaZ, aadB, aacC1, aacC2, aacC3, aacA4, aac6'-IIa, ermA, ermB, ermC, mecA, vanA, vanB, vanC, satA, aac(6')-aph(2''), aad(6'), vat, vga, msrA, sul* and *int* are also objects of this invention.

Diagnostic kits further comprising probes or amplification primers for the detection of any bacterial or fungal species, comprising or not comprising those for the detection of the specific microbial species or genus listed above, and further comprising or not comprising probes and primers for the antibiotic resistance genes listed above, are also objects of this invention.

In a preferred embodiment, such a kit allows for the separate or the simultaneous detection and identification of the above-listed microbial species or genus, antibiotic resistance genes and for the detection of any bacterium.

In the above methods and kits, amplification reactions may include a) polymerase chain reaction (PCR), b) ligase chain reaction, c) nucleic acid sequence-based amplification, d) self-sustained sequence replication, e) strand displacement amplification, f) branched DNA signal amplification, g) transcription-mediated amplification, h) cycling probe technology (CPT) i) nested PCR, or j) multiplex PCR.

In a preferred embodiment, a PCR protocol is used as an amplification reaction.

In a particularly preferred embodiment, a PCR protocol is provided, comprising, for each amplification cycle, an annealing step of 30 seconds at 45-55°C and a denaturation step of only one second at 95°C, without any time allowed specifically for the elongation step. This PCR protocol has been standardized to be suitable for PCR reactions with all selected primer pairs, which greatly facilitates the testing because each clinical sample can be tested with universal, species-specific, genus-specific and antibiotic resistance gene PCR primers under uniform cycling conditions. Furthermore, various combinations of primer pairs may be used in multiplex PCR assays.

We aim at developing a rapid test or kit to discard rapidly all the samples which are negative for bacterial cells and to subsequently detect and identify the above bacterial and/or fungal species and genera and to determine rapidly the bacterial resistance to antibiotics. Although the sequences from the selected antibiotic resistance genes are available from databases and have been used to develop DNA-based tests for their detection, our approach is unique because it represents a major improvement over current gold standard diagnostic methods based on bacterial

cultures. Using an amplification method for the simultaneous bacterial detection and identification and antibiotic resistance genes detection, there is no need for culturing the clinical sample prior to testing. Moreover, a modified PCR protocol has been developed to detect all target DNA sequences in approximately one hour under uniform amplification conditions. This procedure will save lives by optimizing treatment, will diminish antibiotic resistance because less antibiotics will be prescribed, will reduce the use of broad spectrum antibiotics which are expensive, decrease overall health care costs by preventing or shortening hospitalizations, and decrease the time and costs associated with clinical laboratory testing.

In the methods and kits described herein below, the oligonucleotide probes and amplification primers have been derived from larger sequences (i.e. DNA fragments of at least 100 base pairs). All DNA fragments have been obtained either from proprietary fragments or from databases. DNA fragments selected from databases are newly used in a method of detection according to the present invention, since they have been selected for their diagnostic potential.

It is clear to the individual skilled in the art that other oligonucleotide sequences appropriate for (i) the universal bacterial detection, (ii) the detection and identification of the above microbial species or genus and (iii) the detection of antibiotic resistance genes other than those listed in Annex VI may also be derived from the proprietary fragments or selected database sequences. For example, the oligonucleotide primers or probes may be shorter or longer than the ones we have chosen; they may also be selected anywhere else in the proprietary DNA fragments or in the sequences selected from databases; they may be also variants of the same oligonucleotide. If the target DNA or a variant thereof hybridizes to a given oligonucleotide, or if the target DNA or a variant thereof can be amplified by a given oligonucleotide PCR primer pair, the converse is also true; a given target DNA may hybridize to a variant oligonucleotide probe or be amplified by a variant oligonucleotide PCR primer. Alternatively, the oligonucleotides may be designed from any DNA fragment sequences for use in amplification methods other than PCR. Consequently, the core of this invention is the identification of universal, species-specific, genus-specific and resistance gene-specific genomic or non-genomic DNA fragments which are used as a source of specific and ubiquitous oligonucleotide probes and/or amplification primers. Although the selection and evaluation of oligonucleotides suitable for diagnostic purposes requires much effort, it is quite possible for the individual skilled in the art to derive, from the selected DNA fragments, oligonucleotides other than the ones listed in Annex VI which are suitable for diagnostic purposes. When a proprietary fragment or a database sequence is selected for its specificity and ubiquity, it increases the probability that subsets thereof will also be specific and ubiquitous.

Since a high percentage of clinical specimens are negative for bacteria (Table

3), DNA fragments having a high potential for the selection of universal oligonucleotide probes or primers were selected from proprietary and database sequences. The amplification primers were selected from a gene highly conserved in bacteria and fungi, and are used to detect the presence of any bacterial pathogen in clinical specimens in order to determine rapidly (approximately one hour) whether it is positive or negative for bacteria. The selected gene, designated *tuf,* encodes a protein (EF-Tu) involved in the translational process during protein synthesis. The *tuf* gene sequence alignments used to derive the universal primers include both proprietary and database sequences (Example 1 and Annex I). This strategy allows the rapid screening of the numerous negative clinical specimens (around 80% of the specimens received, see Table 3) submitted for bacteriological testing. Tables 4, 5 and 6 provide a list of the bacterial or fungal species used to test the specificity of PCR primers and DNA probes. Table 7 gives a brief description of each species-specific, genus-specific and universal amplification assays which are objects of the present invention. Tables 8, 9 and 10 provide some relevant information about the proprietary and database sequences selected for diagnostic puposes.

DETAILED DESCRIPTION OF THE INVENTION

Development of species-specific, genus-specific, universal and antibiotic resistance gene-specific DNA probes and amplification primers for microorganisms

Selection from databases of sequences suitable for diagnostic purposes

In order to select sequences which are suitable for species-specific or genus-specific detection and identification of bacteria or fungi or, alternatively, for the universal detection of bacteria, the database sequences (GenBank, EMBL and Swiss-Prot) were chosen based on their potential for diagnostic purposes according to sequence information and computer analysis performed with these sequences. Initially, all sequence data available for the targeted microbial species or genus were carefully analyzed. The gene sequences which appeared the most promising for diagnostic purposes based on sequence information and on sequence comparisons with the corresponding gene in other microbial species or genera performed with the Genetics Computer Group (GCG, Wisconsin) programs were selected for testing by PCR. Optimal PCR amplification primers were chosen from the selected database sequences with the help of the Oligo™ 4.0 primer analysis software (National Biosciences Inc., Plymouth, Minn.). The chosen primers were tested in PCR assays for their specificity and ubiquity for the target microbial species or genus. In general, the identification of database sequences from which amplification primers suitable for species-specific or genus-specific detection and identification were selected involved the computer analysis and PCR testing of several candidate gene sequences before

obtaining a primer pair which is specific and ubiquitous for the target microbial species or genus. Annex VI provides a list of selected specific and ubiquitous PCR primer pairs. Annexes I to V and Examples 1 to 4 illustrate the strategy used to select genus-specific, species-specific and universal PCR primers from *tuf* sequences or from the *rec*A gene.

Oligonucleotide primers and probes design and synthesis

The DNA fragments sequenced by us or selected from databases (GenBank and EMBL) were used as sources of oligonucleotides for diagnostic purposes. For this strategy, an array of suitable oligonucleotide primers or probes derived from a variety of genomic DNA fragments (size of more than 100 bp) selected from databases were tested for their specificity and ubiquity in PCR and hybridization assays as described later. It is important to note that the database sequences were selected based on their potential for being species-specific, genus-specific or universal for the detection of bacteria or fungi according to available sequence information and extensive analysis and that, in general, several candidate database sequences had to be tested in order to obtain the desired specificity, ubiquity and sensitivity.

Oligonucleotide probes and amplification primers derived from species-specific fragments selected from database sequences were synthesized using an automated DNA synthesizer (Perkin-Elmer Corp., Applied Biosystems Division). Prior to synthesis, all oligonucleotides (probes for hybridization and primers for DNA amplification) were evaluated for their suitability for hybridization or DNA amplification by polymerase chain reaction (PCR) by computer analysis using standard programs (i.e. the Genetics Computer Group (GCG) programs and the primer analysis software Oligo™ 4.0). The potential suitability of the PCR primer pairs was also evaluated prior to the synthesis by verifying the absence of unwanted features such as long stretches of one nucleotide and a high proportion of G or C residues at the 3' end (Persing *et al.*, 1993, Diagnostic Molecular Microbiology: Principles and Applications, American Society for Microbiology, Washington, D.C.).

The oligonucleotide primers or probes may be derived from either strand of the duplex DNA. The primers or probes may consist of the bases A, G, C, or T or analogs and they may be degenerated at one or more chosen nucleotide position(s). The primers or probes may be of any suitable length and may be selected anywhere within the DNA sequences from proprietary fragments or from selected database sequences which are suitable for (i) the universal detection of bacteria, (ii) the species-specific detection and identification of *Enterococcus faecium*, *Listeria monocytogenes*, *Neisseria meningitidis*, *Staphylococcus saprophyticus*, *Streptococcus agalactiae* and *Candida albicans* (iii) the genus-specific detection of *Streptococcus* species, *Enterococcus* species, *Staphylococcus* species and *Neisseria* species or (iv) the detection of the 26 above-mentioned clinically important antibiotic resistance genes.

Variants for a given target bacterial gene are naturally occurring and are attributable to sequence variation within that gene during evolution (Watson *et al.*, 1987, Molecular Biology of the Gene, 4th ed., The Benjamin/Cummings Publishing Company, Menlo Park, CA; Lewin, 1989, Genes IV, John Wiley & Sons, New York, NY). For example, different strains of the same bacterial species may have a single or more nucleotide variation(s) at the oligonucleotide hybridization site. The person skilled in the art is well aware of the existence of variant bacterial or fungal DNA sequences for a specific gene and that the frequency of sequence variations depends on the selective pressure during evolution on a given gene product. The detection of a variant sequence for a region between two PCR primers may be demonstrated by sequencing the amplification product. In order to show the presence of sequence variants at the primer hybridization site, one has to amplify a larger DNA target with PCR primers outside that hybridization site. Sequencing of this larger fragment will allow the detection of sequence variation at this site. A similar strategy may be applied to show variants at the hybridization site of a probe. Insofar as the divergence of the target sequences or a part thereof does not affect the specificity and ubiquity of the amplification primers or probes, variant bacterial DNA is under the scope of this invention. Variants of the selected primers or probes may also be used to amplify or hybridize to a variant DNA.

Sequencing of *tuf* sequences from a variety of bacterial and fungal species

The nucleotide sequence of a portion of *tuf* genes was determined for a variety of bacterial and fungal species. The amplification primers SEQ ID NOs: 107 and 108, which amplify a *tuf* gene portion of approximately 890 bp, were used for the sequencing of bacterial *tuf* sequences. The amplification primers SEQ ID NOs: 109 and 172, which amplify a *tuf* gene portion of approximately 830 bp, were used for the sequencing of fungal *tuf* sequences. Both primer pairs can amplify *tufA* and *tufB* genes. This is not surprising because these two genes are nearly identical. For example, the entire *tufA* and *tufB* genes from *E. coli* differ at only 13 nucleotide positions (Neidhardt *et al.*, 1996, *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2nd ed., American Society for Microbiology Press, Washington, D.C.). These amplification primers are degenerated at several nucleotide positions and contain inosines in order to allow the amplification of a wide range of *tuf* sequences. The strategy used to select these amplification primers is similar to that illustrated in Annex I for the selection of universal primers. The amplification primers SEQ ID NOs: 107 and 108 could be used to amplify the *tuf* genes from any bacterial species. The amplification primers SEQ ID NOs: 109 and 172 could be used to amplify the *tuf* genes from any fungal species.

The *tuf* genes were amplified directly from bacterial or yeast cultures using the following amplification protocol: One μL of cell suspension was transferred directly to

19 μL of a PCR reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 2.5 mM $MgCl_2$, 1 μM of each of the 2 primers, 200 μM of each of the four dNTPs, 0.5 unit of *Taq* DNA polymerase (Promega Corp., Madison, WI). PCR reactions were subjected to cycling using a MJ Research PTC-200 thermal cycler (MJ Research Inc., Watertown, Mass.) as follows: 3 min at 96°C followed by 30-35 cycles of 1 min at 95°C for the denaturation step, 1 min at 30-50°C for the annealing step and 1 min at 72°C for the extension step. Subsequently, twenty microliters of the PCR-amplified mixture were resolved by electrophoresis in a 1.5% agarose gel. The gel was then visualized by staining with methylene blue (Flores *et al.*, 1992, Biotechniques, **13**:203-205). The size of the amplification products was estimated by comparison with a 100-bp molecular weight ladder. The band corresponding to the specific amplification product (i.e. approximately 890 or 830 bp for bacterial or fungal *tuf* sequences, respectively) was excised from the agarose gel and purified using the QIAquick™ gel extraction kit (QIAGEN Inc., Chatsworth, CA). The gel-purified DNA fragment was then used directly in the sequencing protocol. Both strands of the *tuf* genes amplification product were sequenced by the dideoxynucleotide chain termination sequencing method by using an Applied Biosystems automated DNA sequencer (model 373A) with their PRISM™ Sequenase® Terminator Double-stranded DNA Sequencing Kit (Perkin-Elmer Corp., Applied Biosystems Division, Foster City, CA). The sequencing reactions were all performed by using the amplification primers (SEQ ID NOs: 107 to 109 and 172) and 100 ng per reaction of the gel-purified amplicon. In order to ensure that the determined sequence did not contain errors attributable to the sequencing of PCR artefacts, we have sequenced two preparations of the gel-purified *tuf* amplification product originating from two independent PCR amplifications. For all target microbial species, the sequences determined for both amplicon preparations were identical. Furthermore, the sequences of both strands were 100% complementary thereby confirming the high accuracy of the determined sequence. The *tuf* sequences determined using the above strategy are all in the Sequence Listing (i.e. SEQ ID NOs:118 to 146). Table 13 gives the originating microbial species and the source for each *tuf* sequence in the Sequence Listing.

The alignment of the *tuf* sequences determined by us or selected from databases reveals clearly that the length of the sequenced portion of the *tuf* genes is variable. There may be insertions or deletions of several amino acids. This explains why the size of the sequenced *tuf* amplification product was variable for both bacterial and fungal species. Among the *tuf* sequences determined by our group, we found insertions and deletions adding up to 5 amino acids or 15 nucleotides. Consequently, the nucleotide positions indicated on top of each of Annexes I to V do not correspond for *tuf* sequences having insertions or deletions.

It should also be noted that the various *tuf* sequences determined by us

occasionally contain degenerescences. These degenerated nucleotides correspond to sequence variations between *tufA* and *tufB* genes because the amplification primers amplify both *tuf* genes. These nucleotide variations were not attributable to nucleotide misincorporations by the *taq* DNA polymerase because the sequence of both strands were identical and also because the sequences determined with both preparations of the gel-purified *tuf* amplicons were identical.

The selection of amplification primers from *tuf* sequences

The *tuf* sequences determined by us or selected from databases were used to select PCR primers for (i) the universal detection of bacteria, (ii) the genus-specific detection and identification of *Enterococcus* spp. and *Staphylococcus* spp. and (iii) the species-specific detection and identification of *Candida albicans*. The strategy used to select these PCR primers was based on the analysis of multiple sequence alignments of various *tuf* sequences. For more details about the selection of PCR primers from *tuf* sequences, please refer to Examples 1 to 3 and Annexes I to IV.

The selection of amplification primers from *recA*

The comparison of the nucleotide sequence for the *recA* gene from various bacterial species including 5 species of streptococci allowed the selection of *Streptococcus*-specific PCR primers. For more details about the selection of PCR primers from *recA*, please refer to Example 4 and Annex V.

DNA fragment isolation from *Staphylococcus saprophyticus* by arbitrarily primed PCR

DNA sequences of unknown coding potential for the species-specific detection and identification of *Staphylococcus saprophyticus* were obtained by the method of arbitrarily primed PCR (AP-PCR).

AP-PCR is a method which can be used to generate specific DNA probes for microorganisms (Fani *et al.*, 1993, Mol. Ecol. **2**:243-250). A description of the AP-PCR protocol used to isolate a species-specific genomic DNA fragment from *Staphylococcus saprophyticus* follows. Twenty different oligonucleotide primers of 10 nucleotides in length (all included in the AP-PCR kit OPAD (Operon Technologies, Inc., Alameda, CA)) were tested systematically with DNAs from 3 bacterial strains of *Staphylococcus saprophyticus* (all obtained from the American Type Culture Collection (ATCC): numbers 15305, 35552 and 43867) as well as with DNA from four other staphylococcal species (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 14990, *Staphylococcus haemolyticus* ATCC 29970 and *Staphylococcus hominis* ATCC 35982). For all bacterial species, amplification was performed from a bacterial suspension adjusted to a standard 0.5 McFarland which corresponds to approximately 1.5 x 10^8 bacteria/mL. One μL of the standardized bacterial suspension was transferred directly to 19 μL of a PCR reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 2.5 mM $MgCl_2$,

1.2 μM of only one of the 20 different AP-PCR primers OPAD, 200 μM of each of the four dNTPs and 0.5 unit of *Taq* DNA polymerase (Promega Corp., Madison, WI). PCR reactions were subjected to cycling using a MJ Research PTC-200 thermal cycler (MJ Research Inc.) as follows: 3 min at 96°C followed by 35 cycles of 1 min at 95°C for the denaturation step, 1 min at 32°C for the annealing step and 1 min at 72°C for the extension step. A final extension step of 7 min at 72°C was made after the 35 cycles to ensure complete extension of PCR products. Subsequently, twenty microliters of the PCR amplified mixture were resolved by electrophoresis in a 2% agarose gel containing 0.25 μg/mL of ethidium bromide. The size of the amplification products was estimated by comparison with a 50-bp molecular weight ladder.

Amplification patterns specific for *Staphylococcus saprophyticus* were observed with the AP-PCR primer OPAD-9 (SEQ ID NO: 25). Amplification with this primer consistently showed a band corresponding to a DNA fragment of approximately 450 bp for all *Staphylococcus saprophyticus* strains tested but not for any of the four other staphylococcal species tested. This species-specific pattern was confirmed by testing 10 more clinical isolates of *S. saprophyticus* selected from the culture collection of the microbiology laboratory of the CHUL as well as strains selected from the gram-positive bacterial species listed in Table 5.

The band corresponding to the approximately 450 bp amplicon which was specific and ubiquitous for *S. saprophyticus* based on AP-PCR was excised from the agarose gel and purified using the QIAquick™ gel extraction kit (QIAGEN Inc.). The gel-purified DNA fragment was cloned into the T/A cloning site of the pCR 2.1™ plasmid vector (Invitrogen Inc.) using T4 DNA ligase (New England BioLabs). Recombinant plasmids were transformed into *E. coli* DH5α competent cells using standard procedures. Plasmid DNA isolation was done by the method of Birnboim and Doly (Nucleic Acids Res. **7**:1513-1523) for small-scale preparations. All plasmid DNA preparations were digested with the *Eco*RI restriction endonuclease to ensure the presence of the approximately 450 bp AP-PCR insert into the recombinant plasmids. Subsequently, a large-scale and highly purified plasmid DNA preparation was performed from two selected clones shown to carry the AP-PCR insert by using the QIAGEN plasmid purification kit. These plasmid preparations were used for automated DNA sequencing.

Both strands of the AP-PCR insert from the two selected clones were sequenced by the dideoxynucleotide chain termination sequencing method with SP6 and T7 sequencing primers, by using an Applied Biosystems automated DNA sequencer as described previously. The analysis of the obtained sequences revealed that the DNA sequences for both strands from each clone were 100% complementary. Furthermore, it showed that the entire sequence determined for each clone were both identical. These sequencing data confirm the 100% accuracy for the determined 438

bp sequence (SEQ ID NO: 29). Optimal amplification primers have been selected from the sequenced AP-PCR *Staphylococcus saprophyticus* DNA fragment with the help of the primer analysis software Oligo™ 4.0. The selected primer sequences have been tested in PCR assays to verify their specificity and ubiquity (Table 7). These PCR primers were specific since there was no amplification with DNA from bacterial species other than *S. saprophyticus* selected from Tables 4 and 5. Furthermore, this assay was ubiquitous since 245 of 260 strains of *S. saprophyticus* were efficiently amplified with this PCR assay. When used in combination with another *S. saprophyticus*-specific PCR assay, which is an object of our co-pending U.S. (N.S. 08/526,840) and PCT (PCT/CA/95/00528) patent applications, the ubiquity reaches 100% for these 260 strains.

DNA amplification

For DNA amplification by the widely used PCR (polymerase chain reaction) method, primer pairs were derived from proprietary DNA fragments or from database sequences. Prior to synthesis, the potential primer pairs were analyzed by using the Oligo™ 4.0 software to verify that they are good candidates for PCR amplification.

During DNA amplification by PCR, two oligonucleotide primers binding respectively to each strand of the heat-denatured target DNA from the bacterial genome are used to amplify exponentially *in vitro* the target DNA by successive thermal cycles allowing denaturation of the DNA, annealing of the primers and synthesis of new targets at each cycle (Persing *et al*, 1993, Diagnostic Molecular Microbiology: Principles and Applications, American Society for Microbiology, Washington, D.C.).

Briefly, the PCR protocols were as follow: Treated clinical specimens or standardized bacterial or fungal suspensions (see below) were amplified in a 20 μL PCR reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 2.5 mM $MgCl_2$, 0.4 μM of each primer, 200 μM of each of the four dNTPs and 0.5 unit of *Taq* DNA polymerase (Promega) combined with the TaqStart™ antibody (Clontech Laboratories Inc., Palo Alto, CA). The TaqStart™ antibody, which is a neutralizing monoclonal antibody to *Taq* DNA polymerase, was added to all PCR reactions to enhance the specificity and the sensitivity of the amplifications (Kellogg *et al.*, 1994, Biotechniques **16**:1134-1137). The treatment of the clinical specimens varies with the type of specimen tested, since the composition and the sensitivity level required are different for each specimen type. It consists in a rapid protocol to lyse the bacterial cells and eliminate the PCR inhibitory effects (see example 11 for urine specimen preparation). For amplification from bacterial or fungal cultures, the samples were added directly to the PCR amplification mixture without any pre-treatment step (see example 10). Primer sequences derived from highly conserved regions of the bacterial 16S ribosomal RNA gene were used to provide an internal control for all PCR reactions. Alternatively, the

internal control was derived from sequences not found in microorganisms or in the human genome. The internal control was integrated into all amplification reactions to verify the efficiency of the PCR assays and to ensure that significant PCR inhibition was absent. The internal control derived from rRNA was also useful to monitor the efficiency of bacterial lysis protocols.

PCR reactions were then subjected to thermal cycling (3 min at 95°C followed by 30 cycles of 1 second at 95°C for the denaturation step and 30 second at 55°C for the annealing-extension step) using a PTC-200 thermal cycler (MJ Research Inc.) and subsequently analyzed by standard ethidium bromide-stained agarose gel electrophoresis. The number of cycles performed for the PCR assays varies according to the sensitivity level required. For example, the sensitivity level required for microbial detection directly from clinical specimens is higher for blood specimens than for urine specimens because the concentration of microorganisms associated with a septicemia can be much lower than that associated with a urinary tract infection. Consequently, more sensitive PCR assays having more thermal cycles are required for direct detection from blood specimens. Similarly, PCR assays performed directly from bacterial or fungal cultures may be less sensitive than PCR assays performed directly from clinical specimens because the number of target organisms is normally much lower in clinical specimens than in microbial cultures.

It is clear that other methods for the detection of specific amplification products, which may be faster and more practical for routine diagnosis, may be used. Such methods may be based on the detection of fluorescence after amplification (e.g. TaqMan™ system from Perkin Elmer or Amplisensor™ from Biotronics). Methods based on the detection of fluorescence are particularly promising for utilization in routine diagnosis as they are very rapid, quantitative and can be automated (Example 14).

Microbial pathogens detection and identification may also be performed by solid support or liquid hybridization using species-specific internal DNA probes hybridizing to an amplification product. Such probes may be generated from any species-specific or genus-specific DNA amplification products which are objects of the present invention. Alternatively, the internal probes for species or genus detection and identification may be derived from the amplicons produced by the universal amplification assay. The oligonucleotide probes may be labeled with biotin or with digoxigenin or with any other reporter molecules.

To assure PCR efficiency, glycerol, dimethyl sulfoxide (DMSO) or other related solvents can be used to increase the sensitivity of the PCR and to overcome problems associated with the amplification of a target DNA having a high GC content or forming strong secondary structures (Dieffenbach and Dveksler, 1995, PCR Primer : A Laboratory Manual, Cold Spring Harbor Laboratory Press, Plainview, New York). The

concentration ranges for glycerol and DMSO are 5-15% (v/v) and 3-10% (v/v), respectively. For the PCR reaction mixture, the concentration ranges for the amplification primers and $MgCl_2$ are 0.1-1.5 μM and 1.5-3.5 mM, respectively. Modifications of the standard PCR protocol using external and nested primers (i.e. nested PCR) or using more than one primer pair (i.e. multiplex PCR) may also be used (Persing *et al.*, 1993, Diagnostic Molecular Microbiology: Principles and Applications, American Society for Microbiology, Washington, D.C.). For more details about the PCR protocols and amplicon detection methods, see Examples 9 to 14.

The person skilled in the art of DNA amplification knows the existence of other rapid amplification procedures such as ligase chain reaction (LCR), transcription-mediated amplification (TMA), self-sustained sequence replication (3SR), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), branched DNA (bDNA) and cycling probe technology (CPT) (Lee *et al.*, 1997, Nucleic Acid Amplification Technologies: Application to Disease Diagnosis, Eaton Publishing, Boston, MA ; Persing *et al.*, 1993, Diagnostic Molecular Microbiology: Principles and Applications, American Society for Microbiology, Washington, D.C.). The scope of this invention is not limited to the use of amplification by PCR, but rather includes the use of any rapid nucleic acid amplification method or any other procedure which may be used to increase rapidity and sensitivity of the tests. Any oligonucleotide suitable for the amplification of nucleic acids by approaches other than PCR and derived from the species-specific, genus-specific and universal DNA fragments as well as from selected antibiotic resistance gene sequences included in this document are also under the scope of this invention.

Hybridization assays with oligonucleotide probes

In hybridization experiments, single-stranded oligonucleotides (size less than 100 nucleotides) have some advantages over DNA fragment probes for the detection of bacteria, such as ease of synthesis in large quantities, consistency in results from batch to batch and chemical stability. Briefly, for the hybridizations, oligonucleotides were 5' end-labeled with the radionucleotide γ-^{32}P(dATP) using T4 polynucleotide kinase (Pharmacia) (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The unincorporated radionucleotide was removed by passing the labeled oligonucleotide through a Sephadex G-50™ column. Alternatively, oligonucleotides were labeled with biotin, either enzymatically at their 3' ends or incorporated directly during synthesis at their 5' ends, or with digoxigenin. It will be appreciated by the person skilled in the art that labeling means other than the three above labels may be used.

Each oligonucleotide probe was then tested for its specificity by hybridization to DNAs from a variety of bacterial and fungal species selected from Tables 4, 5 and 6. All of the bacterial or fungal species tested were likely to be pathogens associated

with common infections or potential contaminants which can be isolated from clinical specimens. Each target DNA was released from bacterial cells using standard chemical treatments to lyse the cells (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Subsequently, the DNA was denatured by conventional methods and then irreversibly fixed onto a solid support (e.g. nylon or nitrocellulose membranes) or free in solution. The fixed single-stranded target DNAs were then hybridized with the oligonucleotide probe cells (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Pre-hybridization conditions were in 1 M NaCl + 10% dextran sulfate + 1% SDS + 100 μg/mL salmon sperm DNA at 65°C for 15 min. Hybridization was performed in fresh pre-hybridization solution containing the labeled probe at 65°C overnight. Post-hybridization washing conditions were as follows: twice in 3X SSC containing 1% SDS, twice in 2X SSC containing 1% SDS and twice in 1X SSC containing 1% SDS (all of these washes were at 65°C for 15 min), and a final wash in 0.1X SSC containing 1% SDS at 25°C for 15 min. Autoradiography of washed filters allowed the detection of selectively hybridized probes. Hybridization of the probe to a specific target DNA indicated a high degree of similarity between the nucleotide sequence of these two DNAs because of the high stringency of the washes.

An oligonucleotide probe was considered specific only when it hybridized solely to DNA from the species or genus from which it was isolated. Oligonucleotide probes found to be specific were subsequently tested for their ubiquity (i.e. ubiquitous probes recognized most or all isolates of the target species or genus) by hybridization to microbial DNAs from clinical isolates of the species or genus of interest including ATCC strains. The DNAs from strains of the target species or genus were denatured, fixed onto nylon membranes and hybridized as described above. Probes were considered ubiquitous when they hybridized specifically with the DNA from at least 80% of the isolates of the target species or genus.

Specificity and ubiquity tests for oligonucleotide primers and probes

The specificity of oligonucleotide primers and probes, derived either from the DNA fragments sequenced by us or selected from databases, was tested by amplification of DNA or by hybridization with bacterial or fungal species selected from those listed in Tables 4, 5 and 6, as described in the two previous sections. Oligonucleotides found to be specific were subsequently tested for their ubiquity by amplification (for primers) or by hybridization (for probes) with bacterial DNAs from isolates of the target species or genus. Results for specificity and ubiquity tests with the oligonucleotide primers are summarized in Table 7. The specificity and ubiquity of the PCR assays using the selected amplification primer pairs were tested directly from cultures (see Examples 9 and 10) of bacterial or fungal species.

The various species-specific and genus-specific PCR assays which are objects of the present invention are all specific. For the PCR assays specific to bacterial species or genus, this means that DNA isolated from a wide variety of bacterial species, other than that from the target species or genus and selected from Tables 4 and 5, could not be amplified. For the PCR assay specific to *Candida albicans*, it means there was no amplification with genomic DNA from the fungal species listed in Table 6 as well as with a variety of bacterial species selected from Tables 4 and 5.

The various species-specific and genus-specific PCR assays which are objects of the present invention are also all ubiquitous (Table 7). (i) The species-specific PCR assays for *E. faecium*, *L. monocytogenes*, *S. saprophyticus*, *S. agalactiae* and *C. albicans* amplified genomic DNA from all or most strains of the target species tested, which were obtained from various sources and which are representative of the diversity within each target species (Table 7). The species identification of all of these strains was based on classical biochemical methods which are routinely used in clinical microbiology laboratories. (ii) The genus-specific PCR assays specific for *Enterococcus* spp., *Staphylococcus* spp., *Streptococcus* spp. and *Neisseria* spp. amplified genomic DNA from all or most strains of the target genus tested, which represent all clinically important bacterial species for each target genus. These strains were obtained from various sources and are representative of the diversity within each target genus. Again, the species identification of all of these strains was based on classical biochemical methods which are routinely used in clinical microbiology laboratories. More specifically, the four genus-specific PCR assays amplified the following species: (1) The *Enterococcus*-specific assay amplified efficiently DNA from all of the 11 enterococcal species tested including *E. avium, E. casseliflavus, E. dispar, E. durans, E. faecalis, E. faecium, E. flavescens, E. gallinarum, E. hirae, E. mundtii* and *E. raffinosus*. (2) The *Neisseria*-specific assay amplified efficiently DNA from all of the 12 neisserial species tested including *N. canis, N. cinerea, N. elongata, N. flavescens, N. gonorrhoeae, N. lactamica, N. meningitidis, N. mucosa, N. polysaccharea, N. sicca, N. subflava* and *N. weaveri*. (3) The *Staphylococcus*-specific assay amplified efficiently DNA from 13 of the 14 staphylococcal species tested including *S. aureus, S. auricularis, S. capitis, S. cohnii, S. epidermidis, S. haemolyticus, S. hominis, S. lugdunensis, S. saprophyticus, S. schleiferi, S. simulans, S. warneri* and *S. xylosus*. The staphylococcal species which could not be amplified is *S. sciuri*. (4) Finally, the *Streptococcus*-specific assay amplified efficiently DNA from all of the 22 streptococcal species tested including *S. agalactiae, S. anginosus, S. bovis, S. constellatus, S. crista, S. dysgalactiae, S. equi, S. gordonii, S. intermedius, S. mitis, S. mutans, S. oralis, S. parasanguis, S. pneumoniae, S. pyogenes, S. salivarius, S. sanguis, S. sabrinus, S. suis, S. uberis, S. vestibularis* and *S. viridans*. On the other hand, the *Streptococcus*-specific assay did not amplify 3 out of 9 strains

of *S. mutans* and 1 out of 23 strains of *S. salivarius*, thereby showing a slight lack of ubiquity for these two streptococcal species.

All specific and ubiquitous amplification primers for each target microbial species or genus or antibiotic resistance gene investigated are listed in Annex VI. Divergence in the sequenced DNA fragments can occur, insofar as the divergence of these sequences or a part thereof does not affect the specificity of the probes or amplification primers. Variant bacterial DNA is under the scope of this invention.

The PCR amplification primers listed in Annex VI were all tested for their specificity and ubiquity using reference strains as well as clinical isolates from various geographical locations. The 351 reference strains used to test the amplification and hybridization assays (Tables 4, 5 and 6) were obtained from (i) the American Type Culture Collection (ATCC): 85%, (ii) the Laboratoire de santé publique du Québec (LSPQ): 10%, (iii) the Centers for Disease Control and Prevention (CDC): 3% , (iv) the National Culture Type Collection (NCTC): 1% and (v) several other reference laboratories throughout the world: 1%. These reference strains are representative of (i) 90 gram-negative bacterial species (169 strains; Table 4), (ii) 97 gram-positive bacterial species (154 strains; Table 5) and (iii) 12 fungal species (28 strains; Table 6).

Antibiotic resistance genes

Antimicrobial resistance complicates treatment and often leads to therapeutic failures. Furthermore, overuse of antibiotics inevitably leads to the emergence of bacterial resistance. Our goal is to provide clinicians, in approximately one hour, the needed information to prescribe optimal treatments. Besides the rapid identification of negative clinical specimens with DNA-based tests for universal bacterial detection and the identification of the presence of a specific pathogen in the positive specimens with species- and/or genus-specific DNA-based tests, clinicians also need timely information about the ability of the bacterial pathogen to resist antibiotic treatments. We feel that the most efficient strategy to evaluate rapidly bacterial resistance to antimicrobials is to detect directly from the clinical specimens the most common and clinically important antibiotic resistance genes (i.e. DNA-based tests for the detection of antibiotic resistance genes). Since the sequence from the most important and common bacterial antibiotic resistance genes are available from databases, our strategy was to use the sequence from a portion or from the entire resistance gene to design specific oligonucleotide primers or probes which will be used as a basis for the development of rapid DNA-based tests. The sequence from each of the bacterial antibiotic resistance genes selected on the basis of their clinical relevance (i.e. high incidence and importance) is given in the Sequence Listing. Tables 9 and 10 summarize some characteristics of the selected antibiotic resistance genes. Our approach is unique because the antibiotic resistance genes detection and the bacterial detection and identification are performed simultaneously in multiplex assays under

uniform PCR amplification conditions (Example 13).

Annex VI provides a list of all amplification primers selected from 26 clinically important antibiotic resistance genes which were tested in PCR assays. The various PCR assays for antibiotic resistance genes detection and identification were validated by testing several resistant bacterial isolates known to carry the targeted gene and obtained from various countries. The testing of a large number of strains which do not carry the targeted resistance gene was also performed to ensure that all assays were specific. So far, all PCR assays for antibiotic resistance genes are highly specific and have detected all control resistant bacterial strains known to carry the targeted gene. The results of some clinical studies to validate the array of PCR assays for the detection and identification of antibiotic resistance genes and correlate these DNA-based assays with standard antimicrobials susceptibility testing methods are presented in Tables 11 and 12.

Universal bacterial detection

In the routine microbiology laboratory, a high percentage of clinical specimens sent for bacterial identification are negative by culture (Table 4). Testing clinical samples with universal amplification primers or universal probes to detect the presence of bacteria prior to specific identification and screen out the numerous negative specimens is thus useful as it saves costs and may rapidly orient the clinical management of the patients. Several amplification primers and probes were therefore synthesized from highly conserved portions of bacterial sequences from the *tuf* genes (Table 8). The universal primer selection was based on a multiple sequence alignment constructed with sequences determined by us or selected from available database sequences as described in Example 1 and Annex I.

For the identification of database sequences suitable for the universal detection of bacteria, we took advantage of the fact that the complete genome sequences for two distant microorganisms (i.e. *Mycoplasma genitalium* and *Haemophilus influenzae*) are available. A comparison of the amino acid sequence for all proteins encoded by the genome of these two distant microorganisms led to the identification of highly homologous proteins. An analysis of these homologous proteins allowed to select some promising candidates for the development of universal DNA-based assays for the detection of bacteria. Since the complete nucleotide sequence of several other microbial genomes are presently available in databases, a person skilled in the art could arrive to the same conclusions by comparing genomes sequences other than those of *Mycoplasma genitalium* and *Haemophilus influenzae*. The selected *tuf* gene encodes a protein (EF-Tu) involved in the translation process during protein synthesis. Subsequently, an extensive nucleotide sequence analysis was performed with the *tuf* gene sequences available in databases as well as with novel *tuf* sequences which we have determined as described previously. All computer analysis of amino acid and

nucleotide sequences were performed by using the GCG programs. Subsequently, optimal PCR primers for the universal amplification of bacteria were selected with the help of the Oligo™ program. The selected primers are degenerated at several nucleotide positions and contain several inosines in order to allow the amplification of all clinically relevant bacterial species (Annex I). Inosine is a nucleotide analog able to specifically bind to any of the four nucleotides A, C, G or T. Degenerated oligonucleotides consist of an oligonucleotide mix having two or more of the four nucleotides A, C, G or T at the site of mismatches. The inclusion of inosine and/or of degenerescences in the amplification primers allow mismatch tolerance thereby permitting the amplification of a wider array of target nucleotide sequences (Dieffenbach and Dveksler, 1995 PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Plainview, NY).

The amplification conditions with the universal primers were identical to those used for the species- and genus-specific amplification assays except that the annealing temperature was 50°C instead of 55°C. This universal PCR assay was specific and nearly ubiquitous for the detection of bacteria. The specificity for bacteria was verified by amplifying genomic DNA isolated from the 12 fungal species listed in Table 6 as well as genomic DNA from *Leishmania donovani*, *Saccharomyces cerevisiae* and human lymphocytes. None of the above eukaryotic DNA preparations could be amplified by the universal assay, thereby suggesting that this test is specific for bacteria. The ubiquity of the universal assay was verified by amplifying genomic DNAs from 116 reference strains which represent 95 of the most clinically relevant bacterial species. These species have been selected from the bacterial species listed in Tables 4 and 5. We found that 104 of these 116 strains could be amplified. The bacterial species which could not be amplified belong to the following genera: *Corynebacterium* (11 species) and *Stenotrophomonas* (1 species). Sequencing of the *tuf* genes from these bacterial species has been recently performed. This sequencing data has been used to select new universal primers which may be more ubiquitous. These primers are in the process of being tested. We also observed that for several species the annealing temperature had to be reduced to 45°C in order to get an efficient amplification. These bacterial species include *Gemella morbilbrum*, *Listeria* spp. (3 species) and *Gardnerella vaginalis.* It is important to note that the 95 bacterial species selected from Tables 4 and 5 to test the ubiquity of the universal assay include all of the most clinically relevant bacterial species associated with a variety of human infections acquired in the community or in hospitals (nosocomial infections). The most clinically important bacterial and fungal pathogens are listed in Tables 1 and 2.

EXAMPLES AND ANNEXES

The following examples and annexes are intended to be illustrative of the various methods and compounds of the invention, rather than limiting the scope thereof.

The various annexes show the strategies used for the selection of amplification primers from *tuf* sequences or from the *recA* gene: (i) Annex I illustrates the strategy used for the selection of the universal amplification primers from *tuf* sequences. (ii) Annex II shows the strategy used for the selection of the amplification primers specific for the genus *Enterococcus* from *tuf* sequences. (iii) Annex III illustrates the strategy used for the selection of the amplification primers specific for the genus *Staphylococcus* from *tuf* sequences. (iv) Annex IV shows the strategy used for the selection of the amplification primers specific for the species *Candida albicans* from *tuf* sequences. (v) Annex V illustrates the strategy used for the selection of the amplification primers specific for the genus *Streptococcus* from *recA* sequences. (vi) Annex VI gives a list of all selected primer pairs. As shown in these annexes, the selected amplification primers may contain inosines and/or degenerescences. Inosine is a nucleotide analog able to specifically bind to any of the four nucleotides A, C, G or T. Alternatively, degenerated oligonucleotides which consist of an oligonucleotide mix having two or more of the four nucleotides A, C, G or T at the site of mismatches were used. The inclusion of inosine and/or of degenerescences in the amplification primers allow mismatch tolerance thereby permitting the amplification of a wider array of target nucleotide sequences (Dieffenbach and Dveksler, 1995 PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Plainview, New York).

EXAMPLES

EXAMPLE 1 :

Selection of universal PCR primers from *tuf* sequences. As shown in Annex I, the comparison of *tuf* sequences from a variety of bacterial and eukaryotic species allowed the selection of PCR primers which are universal for the detection of bacteria. The strategy used to design the PCR primers was based on the analysis of a multiple sequence alignment of various *tuf* sequences. This multiple sequence alignment includes *tuf* sequences from 38 bacterial species and 3 eukaryotic species either determined by us or selected from databases (Table 13). A careful analysis of this multiple sequence alignment allowed the selection of primer sequences which are conserved within eubacteria but which discriminate sequences from eukaryotes, thereby permitting the universal detection of bacteria. As shown in Annex I, the selected primers contain several inosines and degenerescences. This was necessary because there is a relatively high polymorphism among bacterial *tuf* sequences despite the fact that this gene is highly conserved. In fact, among the *tuf* sequences that we determined, we found many nucleotide variations as well as some deletions and/or

insertions of amino acids. The selected universal primers were specific and ubiquitous for bacteria (Table 7). Of the 95 most clinically important bacterial species tested, 12 were not amplified. These species belong to the genera *Corynebacterium* (11 species) and *Stenotrophomonas* (1 species). The universal primers did not amplify DNA of non-bacterial origin, including human and other types of eukaryotic DNA.

EXAMPLE 2 :

Selection of genus-specific PCR primers from *tuf* sequences. As shown in Annexes 2 and 3, the comparison of *tuf* sequences from a variety of bacterial species allowed the selection of PCR primers specific for *Enterococcus* spp. or for *Staphylococcus* spp. The strategy used to design the PCR primers was based on the analysis of a multiple sequence alignment of various *tuf* sequences. These multiple sequence alignments include the *tuf* sequences of four representative bacterial species selected from each target genus as well as *tuf* sequences from species of other closely related bacterial genera. A careful analysis of those alignments allowed the selection of oligonucleotide sequences which are conserved within the target genus but which discriminate sequences from other closely related genera, thereby permitting the genus-specific and ubiquitous detection and identification of the target bacterial genus.

For the selection of primers specific for *Enterococcus* spp. (Annex II), we have sequenced a portion of approximately 890 bp of the *tuf* genes for *Enterococcus avium, E. faecalis, E. faecium* and *E. gallinarum*. All other *tuf* sequences used in the alignment were either sequenced by us or selected from databases. The analysis of this sequence alignment led to the selection of a primer pair specific and ubiquitous for *Enterococcus* spp. (Table 7). All of the 11 enterococcal species tested were efficiently amplified and there was no amplification with genomic DNA from bacterial species of other genera.

For the selection of primers specific for *Staphylococcus* spp. (Annex III), we have also sequenced a portion of approximately 890 bp of the *tuf* genes for *Staphylococcus aureus, S. epidermidis, S. saprophyticus* and *S. simulans*. All other *tuf* sequences used in the alignment were either sequenced by us or selected from databases. The analysis of this sequence alignment led to the selection of two primer pairs specific and ubiquitous for *Staphylococcus* spp. (Table 7). Annex III shows the strategy used to select one of these two PCR primer pairs. The same strategy was used to select the other primer pair. Of the 14 staphylococcal species tested, one (*S. sciuri*) could not be amplified by the *Staphylococcus*-specific PCR assays using either one of these two primer pairs. For PCR assays using either one of these two primer pairs, there was no amplification with DNA from species of other bacterial genera.

EXAMPLE 3 :

Selection from *tuf* sequences of PCR primers specific for *Candida albicans*. As shown in Annex IV, the comparison of *tuf* sequences from a variety of bacterial and eukaryotic species allowed the selection of PCR primers specific for *Candida albicans*. The strategy used to design the PCR primers was based on the analysis of a multiple sequence alignment of various *tuf* sequences. This multiple sequence alignment includes *tuf* sequences of five representative fungal species selected from the genus *Candida* which were determined by our group (i.e. *C. albicans, C. glabrata, C. krusei, C. parapsilosis* and *C. tropicalis*) as well as *tuf* sequences from other closely related fungal species. *tuf* sequences from various bacterial species were also included. A careful analysis of this sequence alignment allowed the selection of primers from the *C. albicans tuf* sequence; these primers discriminate sequences from other closely related *Candida* species and other fungal species, thereby permitting the species-specific and ubiquitous detection and identification of *C. albicans* (Table 7). All of 88 *Candida albicans* strains tested were efficiently amplified and there was no amplification with genomic DNA from other fungal or bacterial species.

EXAMPLE 4:

Selection of PCR primers specific for *Streptococcus* from *recA*. As shown in Annex V, the comparison of the various bacterial *recA* gene sequences available from databases (GenBank and EMBL) was used as a basis for the selection of PCR primers which are specific and ubiquitous for the bacterial genus *Streptococcus*. Since sequences of the *recA* gene are available for many bacterial species including five species of streptococci, it was possible to choose sequences well conserved within the genus *Streptococcus* but distinct from the *recA* sequences for other bacterial genera. When there were mismatches between the *recA* gene sequences from the five *Streptococcus* species, an inosine residue was incorporated into the primer (Annex V). The selected primers, each containing one inosine and no degenerescence, were specific and ubiquitous for *Streptococcus* species (Table 7). This PCR assay amplified all of the 22 streptococcal species tested. However, the *Streptococcus*-specific assay did not amplify DNA from 3 out of 9 strains of *S. mutans* and 1 out of 3 strains of *S. salivarius*. There was no amplification with genomic DNA from other bacterial genera (Table 7).

EXAMPLE 5:

Nucleotide sequencing of DNA fragments. The nucleotide sequence of a portion of the *tuf* genes from a variety of bacterial or fungal species was determined by using the dideoxynucleotide chain termination sequencing method (Sanger *et al.*, 1977, Proc. Natl. Acad. Sci. USA. **74**:5463-5467). The sequencing was performed by using an Applied Biosystems automated DNA sequencer (model 373A) with their PRISM™ Sequenase® Terminator Double-stranded DNA Sequencing Kit (Perkin-Elmer Corp.,

Applied Biosystems Division, Foster City, CA). The sequencing strategy does not discriminate *tufA* and *tufB* genes because the sequencing primers hybridize efficiently to both bacterial *tuf* genes. These DNA sequences are shown in the sequence listing (SEQ ID Nos: 118 to 146). The presence of several degenerated nucleotides in the various *tuf* sequences determined by our group (Table 13) corresponds to sequence variations between *tufA* and *tufB*.

Oligonucleotide primers and probes selection. Oligonucleotide probes and amplification primers were selected from the given proprietary DNA fragments or database sequences using the Oligo™ program and were synthesized with an automated ABI DNA synthesizer (Model 391, Perkin-Elmer Corp., Applied Biosystems Division) using phosphoramidite chemistry.

EXAMPLE 6 :

Labeling of oligonucleotides for hybridization assays. Each oligonucleotide was 5' end-labeled with γ-^{32}P (dATP) by the T4 polynucleotide kinase (Pharmacia) as described earlier. The label could also be non-radioactive.

Specificity test for oligonucleotide probes. All labeled oligonucleotide probes were tested for their specificity by hybridization to DNAs from a variety of bacterial and fungal species selected from Tables 4, 5 and 6 as described earlier. Species-specific or genus-specific probes were those hybridizing only to DNA from the microbial species or genus from which it was isolated. Oligonucleotide probes found to be specific were submitted to ubiquity tests as follows.

Ubiquity test for oligonucleotide probes. Specific oligonucleotide probes were then used in ubiquity tests with strains of the target species or genus including reference strains and other strains obtained from various countries and which are representative of the diversity within each target species or genus. Chromosomal DNAs from the isolates were transferred onto nylon membranes and hybridized with labeled oligonucleotide probes as described for specificity tests. The batteries of isolates constructed for each target species or genus contain reference ATCC strains as well as a variety of clinical isolates obtained from various sources. Ubiquitous probes were those hybridizing to at least 80% of DNAs from the battery of clinical isolates of the target species or genus.

EXAMPLE 7:

Same as example 6 except that a pool of specific oligonucleotide probes is used for microbial identification (i) to increase sensitivity and assure 100% ubiquity or (ii) to identify simultaneously more than one microbial species and/or genus. Microbial identification could be performed from microbial cultures or directly from any clinical specimen.

EXAMPLE 8:

Same as example 6 except that bacteria or fungi were detected directly from clinical samples. Any biological sample was loaded directly onto a dot blot apparatus and cells were lysed *in situ* for bacterial or fungal detection and identification. Blood samples should be heparizined in order to avoid coagulation interfering with their convenient loading on a dot blot apparatus.

EXAMPLE 9:

PCR amplification. The technique of PCR was used to increase the sensitivity and the rapidity of the assays. The sets of primers were tested in PCR assays performed directly from bacterial colonies or from a standardized bacterial suspension (see Example 10) to determine their specificity and ubiquity (Table 7). Examples of specific and ubiquitous PCR primer pairs are listed in Annex VI.

Specificity and ubiquity tests for amplification primers. The specificity of all selected PCR primer pairs was tested against DNAs from a variety of bacterial and fungal species selected from Tables 4, 5 and 6 as described earlier. Primer pairs found specific for each species or genus were then tested for their ubiquity to ensure that each set of primers could amplify at least 90% of DNAs from a battery of isolates of the target species or genus. The batteries of isolates constructed for each species contain reference ATCC strains and various clinical isolates from around the world which are representative of the diversity within each species or genus.

Standard precautions to avoid false positive PCR results should be taken (Kwok and Higuchi, 1989, Nature, **239**:237-238). Methods to inactivate PCR amplification products such as the inactivation by uracil-N-glycosylase may be used to control PCR carryover.

EXAMPLE 10:

Amplification directly from bacterial or yeast cultures. PCR assays were performed either directly from a bacterial colony or from a bacterial suspension, the latter being adjusted to a standard McFarland 0.5 (corresponds to approximately 1.5 x 10^8 bacteria/mL). In the case of direct amplification from a colony, a portion of a colony was transferred using a plastic rod directly into a 20 μL PCR reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.4 μM of each primer, 200 μM of each of the four dNTPs and 0.5 unit of *Taq* DNA polymerase (Promega) combined with the TaqStart™ antibody (Clontech Laboratories Inc.). For the bacterial suspension, 1 μL of the cell suspension was added to 19 μL of the same PCR reaction mixture. For the identification from yeast cultures, 1 µL of a standard McFarland 1.0 (corresponds to approximately 3.0 x 10^8 bacteria/mL) concentrated 100 times by centrifugation was added directly to the PCR reaction. This concentration step for yeast cells was performed because a McFarland 0.5 for yeast cells has approximately 200 times fewer cells than a McFarland 0.5 for bacterial cells.

PCR reactions were then subjected to thermal cycling (3 min at 95°C followed by 30 cycles of 1 second at 95°C for the denaturation step and 30 seconds at 55°C for the annealing-extension step) using a PTC-200 thermal cycler. PCR amplification products were then analyzed by standard agarose gel (2%) electrophoresis. Amplification products were visualized in agarose gels containing 0.25 μg/mL of ethidium bromide under UV at 254 nm. The entire PCR assay can be completed in approximately one hour.

Primer sequences derived from highly conserved regions of the bacterial 16S ribosomal RNA gene were used to provide an internal control for all PCR reactions. Alternatively, the internal control was derived from sequences not found in microorganisms or in the human genome. The internal control was integrated into all amplification reactions to verify the efficiency of the PCR assays and to ensure that significant PCR inhibition was absent. The internal control derived from rRNA was also useful to monitor the efficiency of the bacterial lysis protocols. The internal control and the species-specific or genus-specific amplifications were performed simultaneously in multiplex PCR assays.

EXAMPLE 11:

Amplification directly from urine specimens. For PCR amplification performed directly from urine specimens, 1 μL of urine was mixed with 4 μL of a lysis solution containing 500 mM KCl, 100 mM tris-HCl (pH 9.0), 1% triton X-100. After incubation for at least 15 minutes at room temperature, 1 μL of the treated urine specimen was added directly to 19 μL of the PCR reaction mixture. The final concentration of the PCR reagents was 50 mM KCl, 10 mM Tris (pH 9.0), 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.4 μM of each primer, 200 μM of each of the four dNTPs. In addition, each 20 μL reaction contained 0.5 unit of *Taq* DNA polymerase (Promega) combined with the TaqStart™ antibody (Clontech Laboratories Inc.).

Strategies for the internal control, PCR amplification and agarose gel detection of the amplicons are as previously described in example 10.

EXAMPLE 12:

Detection of antibiotic resistance genes. The presence of specific antibiotic resistance genes which are frequently encountered and clinically relevant is identified using the PCR amplification or hybridization protocols described previously. Specific oligonucleotides used as a basis for the DNA-based tests are selected from the antibiotic resistance gene sequences. These tests, which allow the rapid evaluation of bacterial resistance to antimicrobial agents, can be performed either directly from clinical specimens, from a standardized bacterial suspension or from a bacterial colony and should complement diagnostic tests for the universal detection of bacteria as well as for the species-specific and genus-specific microbial detection and identification.

EXAMPLE 13:

Same as examples 10 and 11 except that assays were performed by multiplex PCR (i.e. using several pairs of primers in a single PCR reaction) to reach an ubiquity of 100% for the specific targeted pathogen(s). For more heterogeneous microbial species or genus, a combination of PCR primer pairs may be required to detect and identify all representatives of the target species or genus.

Multiplex PCR assays could also be used to (i) detect simultaneously several microbial species and/or genera or, alternatively, (ii) to simultaneously detect and identify bacterial and/or fungal pathogens and detect specific antibiotic resistance genes either directly from a clinical specimen or from bacterial cultures.

For these applications, amplicon detection methods should be adapted to differentiate the various amplicons produced. Standard agarose gel electrophoresis could be used because it discriminates the amplicons based on their sizes. Another useful strategy for this purpose would be detection using a variety of fluorescent dyes emitting at different wavelengths. The fluorescent dyes can be each coupled with a specific oligonucleotide linked to a fluorescence quencher which is degraded during amplification to release the fluorescent dyes (e.g. TaqMan™, Perkin Elmer).

EXAMPLE 14:

Detection of amplification products. The person skilled in the art will appreciate that alternatives other than standard agarose gel electrophoresis (Example 10) may be used for the revelation of amplification products. Such methods may be based on fluorescence polarization or on the detection of fluorescence after amplification (e.g. Amplisensor™, Biotronics; TaqMan™, Perkin-Elmer Corp.) or other labels such as biotin (SHARP Signal™ system, Digene Diagnostics). These methods are quantitative and may be automated. One of the amplification primers or an internal oligonucleotide probe specific to the amplicon(s) derived from the species-specific, genus-specific or universal DNA fragments is coupled with the fluorescent dyes or with any other label. Methods based on the detection of fluorescence are particularly suitable for diagnostic tests since they are rapid and flexible as fluorescent dyes emitting at different wavelengths are available.

EXAMPLE 15:

Species-specific, genus-specific, universal and antibiotic resistance gene amplification primers can be used in other rapid amplification procedures such as the ligase chain reaction (LCR), transcription-mediated amplification (TMA), self-sustained sequence replication (3SR), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), cycling probe technology (CPT) and branched DNA (bDNA) or any other methods to increase the sensitivity of the test. Amplifications can be performed from isolated bacterial cultures or directly from any clinical specimen. The scope of this invention is therefore not limited to the use of the

DNA sequences from the enclosed Sequence Listing for PCR only but rather includes the use of any procedures to specifically detect bacterial DNA and which may be used to increase rapidity and sensitivity of the tests.

EXAMPLE 16:

A test kit would contain sets of probes specific for each microbial species or genus as well as a set of universal probes. The kit is provided in the form of test components, consisting of the set of universal probes labeled with non-radioactive labels as well as labeled species- or genus-specific probes for the detection of each pathogen of interest in specific types of clinical samples. The kit will also include test reagents necessary to perform the pre-hybridization, hybridization, washing steps and hybrid detection. Finally, test components for the detection of known antibiotic resistance genes (or derivatives therefrom) will be included. Of course, the kit will include standard samples to be used as negative and positive controls for each hybridization test.

Components to be included in the kits will be adapted to each specimen type and to detect pathogens commonly encountered in that type of specimen. Reagents for the universal detection of bacteria will also be included. Based on the sites of infection, the following kits for the specific detection of pathogens may be developed:

- A kit for the universal detection of bacterial or fungal pathogens from all clinical specimens which contains sets of probes specific for highly conserved regions of the microbial genomes.

- A kit for the detection of microbial pathogens retrieved from urine samples, which contains 5 specific test components (sets of probes for the detection of *Enterococcus faecium, Enteroccus* species, *Staphylococcus saprophyticus, Staphylococcus* species and *Candida albicans)*.

- A kit for the detection of respiratory pathogens which contains 3 specific test components (sets of probes for the detection of *Staphylococcus* species, *Enterococcus* species and *Candida albicans*).

- A kit for the detection of pathogens retrieved from blood samples, which contains 10 specific test components (sets of probes for the detection of *Streptococcus* species, *Streptococcus agalactiae*, *Staphylococcus* species, *Staphylococcus saprophyticus*, *Enterococcus* species, *Enterococcus faecium*, *Neisseria* species, *Neisseria meningitidis*, *Listeria monocytogenes* and *Candida albicans)*. This kit can also be applied for direct detection and identification from blood cultures.

- A kit for the detection of pathogens causing meningitis, which contains 5 specific test components (sets of probes for the detection of *Streptococcus* species, *Listeria monocytogenes, Neisseria meningitidis, Neisseria* species and *Staphylococcus* species).

- A kit for the detection of clinically important antibiotic resistance genes which contains sets of probes for the specific detection of at least one of the 26 following genes associated with antibiotic resistance: *bla_{tem}*, *bla_{rob}*, *bla_{shv}*, *bla_{oxa}*, *blaZ, aadB, aacC1, aacC2, aacC3, aacA4, aac6'-IIa, ermA, ermB, ermC, mecA, vanA, vanB, vanC, satA, aac(6')-aph(2"), aad(6'), vat, vga, msrA, sul* and *int.*

- Other kits adapted for the detection of pathogens from skin, abdominal wound or any other clinically relevant infections may also be developed.

EXAMPLE 17:

Same as example 16 except that the test kits contain all reagents and controls to perform DNA amplification assays. Diagnostic kits will be adapted for amplification by PCR (or other amplification methods) performed directly either from clinical specimens or from microbial cultures. Components required for (i) universal bacterial detection, (ii) species-specific and genus-specific bacterial and/or fungal detection and identification and (iii) detection of antibiotic resistance genes will be included.

Amplification assays could be performed either in tubes or in microtitration plates having multiple wells. For assays in plates, the wells will contain the specific amplification primers and control DNAs and the detection of amplification products will be automated. Reagents and amplification primers for universal bacterial detection will be included in kits for tests performed directly from clinical specimens. Components required for species-specific and genus-specific bacterial and/or fungal detection and identification as well as for the simultaneous antibiotic resistance genes detection will be included in kits for testing directly from bacterial or fungal cultures as well as in kits for testing directly from any type of clinical specimen.

The kits will be adapted for use with each type of specimen as described in example 16 for hybridization-based diagnostic kits.

EXAMPLE 18:

It is understood that the use of the probes and amplification primers described in this invention for bacterial and/or fungal detection and identification is not limited to clinical microbiology applications. In fact, we feel that other sectors could also benefit from these new technologies. For example, these tests could be used by industries for quality control of food, water, air, pharmaceutical products or other products requiring microbiological control. These tests could also be applied to detect and identify bacteria or fungi in biological samples from organisms other than humans (e.g. other primates, birds, plants, mammals, farm animals, livestock and others). These diagnostic tools could also be very useful for research purposes including clinical trials and epidemiological studies.

This invention has been described herein above, and it is readily apparent that modifications can be made thereto without departing from the spirit of this invention. These modifications are under the scope of this invention, as defined in the appended claims.

Table 1. Distribution (%) of nosocomial pathogens for various human infections in USA (1990-1992)[1].

Pathogen	UTI[2]	SSI[3]	BSI[4]	Pneumonia	CSF[5]
Escherichia coli	27	9	5	4	2
Staphylococcus aureus	2	21	17	21	2
Staphylococcus epidermidis	2	6	20	0	1
Enterococcus faecalis	16	12	9	2	0
Enterococcus faecium	1	1	0	0	0
Pseudomonas aeruginosa	12	9	3	18	0
Klebsiella pneumoniae	7	3	4	9	0
Proteus mirabilis	5	3	1	2	0
Streptococcus pneumoniae	0	0	3	1	18
Group B *Streptococci*	1	1	2	1	6
Other *Streptococci*	3	5	2	1	3
Haemophilus influenzae	0	0	0	6	45
Neisseria meningitidis	0	0	0	0	14
Listeria monocytogenes	0	0	0	0	3
Other *Enterococci*	1	1	0	0	0
Other *Staphylococci*	2		8	13	20
Candida albicans	9	3	5	5	0
Other *Candida*	2		1	3	10
Enterobacter spp.	5	7	4	12	2
Acinetobacter spp.	1	1	2	4	2
Citrobacter spp.	2	1	1	1	0
Serratia marcescens	1	1	1	3	1
Other *Klebsiella*	1	1	1	2	1
Others	0	6	4	5	0

[1] Data recorded by the National Nosocomial Infections Surveillance (NNIS) from 80 hospitals (Emori and Gaynes, 1993, Clin. Microbiol. Rev., **6**:428-442).

[2] Urinary tract infection.

[3] Surgical site infection.

[4] Bloodstream infection.

[5] Cerebrospinal fluid.

Table 2. **Distribution (%) of bloodstream infection pathogens in Quebec (1995), Canada (1992), UK (1969-1988) and USA (1990-1992).**

Organism	Quebec[1]	Canada[2]	UK[3]		USA[4]
			Community-acquired	Hospital-acquired	Hospital-acquired
E. coli	15.6	53.8	24.8	20.3	5.0
S. epidermidis and other CoNS[5]	25.8	NI[6]	0.5	7.2	31.0
S. aureus	9.6	NI	9.7	19.4	16.0
S. pneumoniae	6.3	NI	22.5	2.2	NR[7]
E. faecalis	3.0	NI	1.0	4.2	NR
E. faecium	2.6	NI	0.2	0.5	NR
Enterococcus spp.	NR	NI	NR	NR	9.0
H. influenzae	1.5	NR	3.4	0.4	NR
P. aeruginosa	1.5	8.2	1.0	8.2	3.0
K. pneumoniae	3.0	11.2	3.0	9.2	4.0
P. mirabilis	NR	3.9	2.8	5.3	1.0
S. pyogenes	NR	NI	1.9	0.9	NR
Enterobacter spp.	4.1	5.5	0.5	2.3	4.0
Candida spp.	8.5	NI	NR	1.0	8.0
Others	18.5	17.4[8]	28.7	18.9	19.0

[1] Data obtained for 270 isolates collected at the Centre Hospitalier de l'Université Laval (CHUL) during a 5 month period (May to October 1995).

[2] Data from 10 hospitals throughout Canada representing 941 gram-negative bacterial isolates. (Chamberland *et al.*, 1992, Clin. Infect. Dis., **15**:615-628).

[3] Data from a 20-year study (1969-1988) for nearly 4000 isolates (Eykyn *et al.*, 1990, J. Antimicrob. Chemother., Suppl. C, **25**:41-58).

[4] Data recorded by the National Nosocomial Infections Surveillance (NNIS) from 80 hospitals (Emori and Gaynes, 1993, Clin. Microbiol. Rev., **6**:428-442).

[5] Coagulase-negative staphylococci.

[6] NI, not included. This survey included only gram-negative species.

[7] NR, incidence not reported for these species or genera.

[8] In this case, 17.4 stands for other gram-negative bacterial species.

Table 3. Distribution of positive and negative clinical specimens tested at the microbiology laboratory of the CHUL (February 1994 – January 1995).

Clinical specimens and/or sites	No. of samples tested (%)	% of positive specimens	% of negative specimens
Urine	17,981 (54.5)	19.4	80.6
Blood culture/marrow	10,010 (30.4)	6.9	93.1
Sputum	1,266 (3.8)	68.4	31.6
Superficial pus	1,136 (3.5)	72.3	27.7
Cerebrospinal fluid	553 (1.7)	1.0	99.0
Synovial fluid	523 (1.6)	2.7	97.3
Respiratory tract	502 (1.5)	56.6	43.4
Deep pus	473 (1.4)	56.8	43.2
Ears	289 (0.9)	47.1	52.9
Pleural and pericardial fluid	132 (0.4)	1.0	99.0
Peritoneal fluid	101(0.3)	28.6	71.4
Total:	**32,966 (100.0)**	**20.0**	**80.0**

Table 4. Gram-negative bacterial species (90) used to test the specificity of PCR primers and DNA probes (continues on next page).

	Bacterial species	Number of reference strains tested[a]	Bacterial species	Number of reference strains tested[a]
5	*Acinetobacter baumannii*	1	*Moraxella phenylpyruvica*	1
	Acinetobacter lwoffii	3	*Morganella morganii*	1
	Actinobacillus lignieresii	1	*Neisseria animalis*	1
	Alcaligenes faecalis	1	*Neisseria canis*	1
	Alcaligenes odorans	1	*Neisseria caviae*	1
10	*Alcaligenes xylosoxydans*		*Neisseria cinerea*	1
	subsp. *denitrificans*	1	*Neisseria cuniculi*	1
	Bacteroides distasonis	1	*Neisseria elongata*	1
			subsp. *elongata*	
	Bacteroides fragilis	1	*Neisseria elongata*	1
			subsp. *glycoytica*	
	Bacteroides ovatus	1	*Neisseria flavescens*	1
15	*Bacteroides thetaiotaomicron*	1	*Neisseria flavescens* Branham	1
	Bacteroides vulgatus	1	*Neisseria gonorrhoeae*	18
	Bordetella bronchiseptica	1	*Neisseria lactamica*	1
	Bordetella parapertussis	1	*Neisseria meningitidis*	4
20	*Bordetella pertussis*	2	*Neisseria mucosa*	2
	Burkholderia cepacia	1	*Neisseria polysaccharea*	1
	Citrobacter amalonaticus	1	*Neisseria sicca*	3
	Citrobacter diversus	2	*Neisseria subflava*	3
	subsp. *koseri*			
25	*Citrobacter freundii*	1	*Neisseria weaveri*	1
	Comamonas acidovorans	1	*Ochrobactrum antropi*	1
	Enterobacter aerogenes	1	*Pasteurella aerogenes*	1
	Enterobacter agglomerans	1	*Pasteurella multocida*	1
30	*Enterobacter cloacae*	1	*Prevotella melaninogenica*	1
	Escherichia coli	9	*Proteus mirabilis*	3
	Escherichia fergusonii	1	*Proteus vulgaris*	1

Bacterial species	Number of reference strains tested[a]	Bacterial species	Number of reference strains tested[a]
Escherichia hermannii	1	*Providencia alcalifaciens*	1
Escherichia vulneris	1	*Providencia rettgeri*	1
Flavobacterium meningosepticum	1	*Providencia rustigianii*	1
Flavobacterium indologenes	1	*Providencia stuartii*	1
Flavobacterium odoratum	1	*Pseudomonas aeruginosa*	14
Fusobacterium necrophorum	2	*Pseudomonas fluorescens*	2
Gardnerella vaginalis	1	*Pseudomonas stutzeri*	1
Haemophilus haemolyticus	1	*Salmonella arizonae*	1
Haemophilus influenzae	12	*Salmonella choleraesuis*	1
Haemophilus parahaemolyticus	1	*Salmonella gallinarum*	1
Haemophilus parainfluenzae	2	*Salmonella typhimurium*	3
Hafnia alvei	1	*Serratia liquefaciens*	1
Kingella indologenes subsp. *suttonella*	1	*Serratia marcescens*	1
Kingella kingae	1	*Shewanella putida*	1
Klebsiella ornithinolytica	1	*Shigella boydii*	1
Klebsiella oxytoca	1	*Shigella dysenteriae*	1
Klebsiella pneumoniae	8	*Shigella flexneri*	1
Moraxella atlantae	1	*Shigella sonnei*	1
Moraxella catarrhalis	5	*Stenotrophomonas maltophilia*	1
Moraxella lacunata	1	*Yersinia enterocolitica*	1
Moraxella osloensis	1		

[a] Most reference strains were obtained from the American Type Culture Collection (ATCC). The other reference strains were obtained from (i) the Laboratoire de Santé Publique du Québec (LSPQ), (ii) the Center for Disease Control and Prevention (CDC) and (iii) the National Culture Type Collection (NCTC).

Table 5. Gram-positive bacterial species (97) used to test the specificity of PCR primers and DNA probes (continues on next page).

Bacterial species	Number of reference strains tested[a]	Bacterial species	Number of reference strains tested[a]
Abiotrophia adiacens	1	*Micrococcus kristinae*	1
Abiotrophia defectiva	1	*Micrococcus luteus*	1
Actinomyces israelii	1	*Micrococcus lylae*	1
Clostridium perfringens	1	*Micrococcus roseus*	1
Corynebacterium accolens	1	*Micrococcus varians*	1
Corynebacterium aquaticum	1	*Peptococcus niger*	1
Corynebacterium bovis	1	*Peptostreptococcus anaerobius*	1
Corynebacterium cervicis	1	*Peptostreptococcus asaccharolyticus*	1
Corynebacterium diphteriae	6	*Staphylococcus aureus*	10
Corynebacterium flavescens	1	*Staphylococcus auricularis*	1
Corynebacterium genitalium	6	*Staphylococcus capitis* subsp. *urealyticus*	1
Corynebacterium jeikeium	1	*Staphylococcus cohnii*	1
Corynebacterium kutcheri	1	*Staphylococcus epidermidis*	2
Corynebacterium matruchotii	1	*Staphylococcus haemolyticus*	2
Corynebacterium minutissimum	1	*Staphylococcus hominis*	2
Corynebacterium mycetoides	1	*Staphylococcus lugdunensis*	1
Corynebacterium pseudodiphtheriticum	1	*Staphylococcus saprophyticus*	3
Corynebacterium pseudogenitalium	6	*Staphylococcus schleiferi*	1
Corynebacterium renale	1	*Staphylococcus sciuri*	1
Corynebacterium striatum	1	*Staphylococcus simulans*	1
Corynebacterium ulcerans	1	*Staphylococcus warneri*	1

Bacterial species	Number of reference strains tested[a]	Bacterial species	Number of reference strains tested[a]
Corynebacterium urealyticum	1	*Staphylococcus xylosus*	1
Corynebacterium xerosis	1	*Streptococcus agalactiae*	6
Enterococcus avium	1	*Streptococcus anginosus*	2
Enterococcus casseliflavus	1	*Streptococcus bovis*	2
Enterococcus cecorum	1	*Streptococcus constellatus*	1
Enterococcus dispar	1	*Streptococcus crista*	1
Enterococcus durans	1	*Streptococcus dysgalactiae*	1
Enterococcus faecalis	6	*Streptococcus equi*	1
Enterococcus faecium	3	*Streptococcus gordonii*	1
Enterococcus flavescens	1	*Group C Streptococci*	1
Enterococcus gallinarum	3	*Group D Streptococci*	1
Enterococcus hirae	1	*Group E Streptococci*	1
Enterococcus mundtii	1	*Group F Streptococci*	1
Enterococcus pseudoavium	1	*Group G Streptococci*	1
Enterococcus raffinosus	1	*Streptococcus intermedius*	1
Enterococcus saccharolyticus	1	*Streptococcus mitis*	2
Enterococcus solitarius	1	*Streptococcus mutans*	1
Eubacterium lentum	1	*Streptococcus oralis*	1
Gemella haemolysans	1	*Streptococcus parasanguis*	1
Gemella morbillorum	1	*Streptococcus pneumoniae*	6
Lactobacillus acidophilus	1	*Streptococcus pyogenes*	3
Listeria innocua	1	*Streptococcus salivarius*	2
Listeria ivanovii	1	*Streptococcus sanguis*	2
Listeria grayi	1	*Streptococcus sobrinus*	1
Listeria monocytogenes	3	*Streptococcus suis*	1
Listeria murrayi	1	*Streptococcus uberis*	1
Listeria seeligeri	1	*Streptococcus vestibularis*	1
Listeria welshimeri	1		

[a] Most reference strains were obtained from the American Type Culture Collection (ATCC). The other reference strains were obtained from (i) the Laboratoire de Santé Publique du Québec (LSPQ), (ii) the Center for Disease Control and Prevention (CDC) and (iii) the National Culture Type Collection (NCTC).

Table 6. Fungal species (12) used to test the specificity of PCR primers and DNA probes.

Fungal species	Number of reference strains tested[a]
Candida albicans	12
Candida glabrata	1
Candida guilliermondii	1
Candida kefyr	3
Candida krusei	2
Candida lusitaniae	1
Candida parapsilosis	2
Candida tropicalis	3
Rhodotorula glutinis	1
Rhodotorula minuta	1
Rhodotorula rubra	1
Saccharomyces cerevisiae	1

[a] Most reference strains were obtained from (i) the American Type Culture Collection (ATCC) and (ii) the Laboratoire de Santé Publique du Québec (LSPQ).

Table 7. PCR assays developed for several clinically important bacterial and fungal pathogens (continues on next page).

Organism	Primer Pair[a] SEQ ID NO	Amplicon size (bp)	Ubiquity[b]	DNA amplification from culture[c]	DNA amplification from specimens[d]
Enterococcus faecium	1-2	216	79/80	+	+
Listeria monocytogenes	3-4	130	164/168[e]	+	+
Neisseria meningitidis	5-6	177	258/258	+	+
Staphylococcus saprophyticus	7-8	149	245/260	+	NT
Streptococcus agalactiae	9-10	154	29/29	+	+
Candida albicans	11-12	149	88/88	+	NT
Enterococcus spp. (11 species)[f]	13-14	112	87/87	+	NT
Neisseria spp. (12 species)[f]	15-16	103	321/321	+	+
Staphylococcus spp. (14 species)	17-18	192	13/14	+	NT
	19-20	221	13/14	+	NT
Streptococcus spp. (22 species)[f]	21-22	153	210/214[g]	+	+
Universal detection[h] (95 species)[i]	23-24	309	104/ 116[i]	+	+

[a] All primer pairs are specific in PCR assays since no amplification was observed with DNA from the bacterial and fungal species other than the species of interest listed in Tables 4, 5 and 6.

[b] Ubiquity was tested by using reference strains as well as strains from throughout the world, which are representatite of the diversity within each target species or genus.

[c] For all primer pairs, PCR amplifications performed directly from a standardized microbial suspension (MacFarland) or from a colony were all specific and ubiquitous.

[d] PCR assays performed directly from blood cultures, urine specimens or

cerebrospinal fluid. NT, not tested.

[e] The four *L. monocytogenes* strains undetected are not clinical isolates. These strains were isolated from food and are not associated with a human infection.

[f] The bacterial species tested include all those clinically relevant for each genus (Tables 4 and 5). All of these species were efficiently amplified by their respective genus-specific PCR assay, except for the *Staphylococcus*-specific assay, which does not amplify *S. sciuri*.

[g] The *Streptococcus*-specific PCR assay did not amplify 3 out of 9 strains of *S. mutans* and 1 out of 3 strains of *S. salivarius*.

[h] The primers selected for universal bacterial detection do not amplify DNA of non-bacterial origin, including human and other types of eukaryotic genomic DNA.

[i] For the universal amplification, the 95 bacterial species tested represent the most clinically important bacterial species listed in Tables 4 and 5. The 12 strains not amplified are representatives of genera *Corynebacterium* (11 species) and *Stenotrophomonas* (1 species).

Table 8. Target genes for the various genus-specific, species-specific and universal amplification assays.

Microorganisms	Gene	Protein encoded
Candida albicans	*tuf*	translation elongation factor EF-Tu
Enterococcus faecium	*ddl*	D-alanine:D-alanine ligase
Listeria monocytogenes	*actA*	actin-assembly inducing protein
Neisseria meningitidis	*omp*	outer membrane protein
Streptococcus agalactiae	*cAMP*	cAMP factor
Staphylococcus saprophyticus	unknown	unknown
Enterococcus spp.	*tuf*	translation elongation factor EF-Tu
Neisseria spp.	*asd*	ASA-dehydrogenase
Staphylococcus spp.	*tuf*	translation elongation factor EF-Tu
Streptococcus spp.	*recA*	RecA protein
Universal detection	*tuf*	translation elongation factor EF-Tu

Table 9. Antibiotic resistance genes selected for diagnostic purposes.

Genes	SEQ ID NOs		Antibiotics	Bacteria[a]
	selected primers	originating fragment		
bla$_{oxa}$	49-50	110	β-lactams	*Enterobacteriaceae*, *Pseudomonadaceae*
blaZ	51-52	111	β-lactams	*Enterococcus* spp.
aac6'-IIa	61-64	112	Aminoglycosides	*Pseudomonadaceae*
ermA	91-92	113	Macrolides	*Staphylococcus* spp.
ermB	93-94	114	Macrolides	*Staphylococcus* spp.
ermC	95-96	115	Macrolides	*Staphylococcus* spp.
vanB	71-74	116	Vancomycin	*Enterococcus* spp.
vanC	75-76	117	Vancomycin	*Enterococcus* spp.
aad(6')	173-174	-	Streptomycin	*Enterococcus* spp.

[a] Bacteria having high incidence for the specified antibiotic resistance genes. The presence of these antibiotic resistance genes in other bacteria is not excluded.

Table 10. Antibiotic resistance genes from our co-pending US (N.S. 08/526840) and PCT (PCT/CA/95/00528) patent applications for which we have selected PCR primer pairs.

Genes	SEQ ID NOs of selected primers	Antibiotics	Bacteria[a]
bla$_{tem}$	37-40	β-lactams	*Enterobacteriaceae*, *Pseudomonadaceae*, *Haemophilus* spp., *Neisseria* spp.
bla$_{rob}$	45-48	β-lactams	*Haemophilus* spp., *Pasteurella* spp.
bla$_{shv}$	41-44	β-lactams	*Klebsiella* spp. and other *Enterobacteriaceae*
*aad*B	53-54	Aminoglycosides	*Enterobacteriaceae*, *Pseudomonadaceae*
*aac*C1	55-56		
*aac*C2	57-58		
*aac*C3	59-60		
*aac*A4	65-66		
*mec*A	97-98	β-lactams	*Staphylococcus* spp.
*van*A	67-70	Vancomycin	*Enterococcus* spp.
*sat*A	81-82	Macrolides	*Enterococcus* spp.
aac(6')-*aph*(2")	83-86	Aminoglycosides	*Enterococcus* spp., *Staphylococcus* spp.
vat	87-88	Macrolides	*Staphylococcus* spp.
vga	89-90	Macrolides	*Staphylococcus* spp.
*msr*A	77-80	Erythromycin	*Staphylococcus* spp.
int	99-102	β-lactams, trimethoprim, aminoglycosides, antiseptic, chloramphenicol	*Enterobacteriaceae*, *Pseudomonadaceae*
sul	103-106		

[a] Bacteria having high incidence for the specified antibiotic resistance genes. The presence of these antibiotic resistance genes in other bacteria is not excluded.

Table 11. Correlation between disk diffusion and PCR amplification of antibiotic resistance genes in *Staphylococcus* species[a].

				Disk diffusion (Kirby-Bauer)[b]		
Antibiotic	Phenotype	PCR	Resistant	Intermediate	Sensitive
Penicillin	*blaZ*	+	165	0	0
		-	0	0	31
Oxacillin	*mecA*	+	51	11	4
		-	2	0	128
Gentamycin	*aac*(6')*aph*(2")	+	24	18	6
		-	0	0	148
Erythromycin	*ermA*	+	15	0	0
	ermB	+	0	0	0
	ermC	+	43	0	0
	msrA	+	4	0	0
		-	0	1	136

[a] The *Staphylococcus* strains studied include *S. aureus* (82 strains), *S. epidermidis* (83 strains), *S. hominis* (2 strains), *S. capitis* (3 strains), *S. haemolyticus* (9 strains), *S. simulans* (12 strains) and *S. warneri* (5 strains), for a total of 196 strains.

[b] Susceptibility testing was performed by the method of Kirby-Bauer according to the protocol reccommended by the National Committee of Clinical Laboratory Standards (NCCLS).

Table 12. Correlation between disk diffusion profiles and PCR amplification of antibiotic resistance genes in *Enterococcus* species[a].

Antibiotic	Phenotype	PCR	Disk diffusion (Kirby-Bauer)[b]	
			Resistant	Sensitive
Ampicillin	*blaZ*	+	0	2
		-	1	30
Gentamycin	*aac*(6')*aph*(2")	+	51	1
		-	3	38
Streptomycin	*aad*(6')	+	26	15
		-	6	27
Vancomycin	*vanA*	+	36	0
	vanB	+	26	0
		-	0	40

[a] The *Enterococcus* strains studied include *E. faecalis* (33 strains) and *E. faecium* (69 strains), for a total of 102 strains.

[b] Susceptibility testing was performed by the method of Kirby-Bauer according to the protocol reccommended by the National Committee of Clinical Laboratory Standards (NCCLS).

Table 13. Origin of *tuf* sequences in the Sequence Listing (continues on next page).

SEQ ID NO	Bacterial or fungal species	Source
118	*Abiotrophia adiacens*	This patent
119	*Abiotrophia defectiva*	This patent
120	*Candida albicans*	This patent
121	*Candida glabrata*	This patent
122	*Candida krusei*	This patent
123	*Candida parapsilosis*	This patent
124	*Candida tropicalis*	This patent
125	*Corynebacterium accolens*	This patent
126	*Corynebacterium diphteriae*	This patent
127	*Corynebacterium genitalium*	This patent
128	*Corynebacterium jeikeium*	This patent
129	*Corynebacterium pseudotuberculosis*	This patent
130	*Corynebacterium striatum*	This patent
131	*Enterococcus avium*	This patent
132	*Enterococcus faecalis*	This patent
133	*Enterococcus faecium*	This patent
134	*Enterococcus gallinarum*	This patent
135	*Gardnerella vaginalis*	This patent
136	*Listeria innocua*	This patent
137	*Listeria ivanovii*	This patent
138	*Listeria monocytogenes*	This patent
139	*Listeria seeligeri*	This patent
140	*Staphylococcus aureus*	This patent
141	*Staphylococcus epidermidis*	This patent
142	*Staphylococcus saprophyticus*	This patent
143	*Staphylococcus simulans*	This patent
144	*Streptococcus agalactiae*	This patent
145	*Streptococcus pneumoniae*	This patent

SEQ ID NO	Bacterial or fungal species	Source
146	*Streptococcus salivarius*	This patent
147	*Agrobacterium tumefaciens*	Database
148	*Bacillus subtilis*	Database
149	*Bacteroides fragilis*	Database
150	*Borrelia burgdorferi*	Database
151	*Brevibacterium linens*	Database
152	*Burkholderia cepacia*	Database
153	*Chlamydia trachomatis*	Database
154	*Escherichia coli*	Database
155	*Fibrobacter succinogenes*	Database
156	*Flavobacterium ferrugineum*	Database
157	*Haemophilus influenzae*	Database
158	*Helicobacter pylori*	Database
159	*Micrococcus luteus*	Database
160	*Mycobacterium tuberculosis*	Database
161	*Mycoplasma genitalium*	Database
162	*Neisseria gonorrhoeae*	Database
163	*Rickettsia prowazekii*	Database
164	*Salmonella typhimurium*	Database
165	*Shewanella putida*	Database
166	*Stigmatella aurantiaca*	Database
167	*Streptococcus pyogenes*	Database
168	*Thiobacillus cuprinus*	Database
169	*Treponema pallidum*	Database
170	*Ureaplasma urealyticum*	Database
171	*Wolinella succinogenes*	Database

Annex I: **Strategy for the selection from *tuf* sequences of the universal amplification primers (continues on pages 49 to 51).**

	491	517...776	802	SEQ ID NO
Abiotrophia adiacens	CAACTGTAAC	TGGTGTTGAA	ATGTTCC...AAATGGT	AATGCCTGGT GATAACGTAA	118
Abiotrophia defectiva	CTACCGTTAC	CGGTGTTGAA	ATGTTCC...AAATGGT	TATGCCAGGC GACAACGTAC	119
Agrobacterium tumefaciens	CGACTGTTAC	CGGCGTTGAA	ATGTTCC...AAATGGT	TATGCCTGGC GACAACGTCA	147
Bacillus subtilis	CAACTGTTAC	AGGTGTTGAA	ATGTTCC...AAATGGT	TATGCCTGGA GATAACACTG	148
Bacteroides fragilis	CAGTTGTAAC	AGGTGTTGAA	ATGTTCC...AAATGGT	AATGCCGGGT GATAACGTAA	149
Borrelia burgdorferi	CTACTGTTAC	TGGTGTTGAA	ATGTTCC...AAATGGT	TATGCCTGGT GATAATGTTG	150
Brevibacterium linens	CGACTGTCAC	CGCTATCGAG	ATGTTCC...AGATGGT	CATGCCCGGC GACACCACCG	151
Burkholderia cepacia	CGACCTGCAC	GGGCGTTGAA	ATGTTCC...AAATGGT	CATGCCGGGC GACAACGTGT	152
Chlamydia trachomatis	CGATTGTTAC	TGGGGTTGAA	ATGTTCA...AGATGGT	CATGCCTGGG GATAACGTTG	153
Corynebacterium diphteriae	CCACCGTTAC	CGGTATCGAG	ATGTTCC...AGATGGT	CATGCCTGGC GACAACGTCG	126

Corynebacterium genitalium	CCACCGTTAC CTCCATCGAG ATGTTCA...AGATGGT TATGCCGGGC GACAACGTTG	127
Corynebacterium jeikeium	CCACCGTTAC CTCCATCGAG ATGTTCA...AGATGGT TATGCCGGGC GACAACGTTG	128
Enterococcus faecalis	CAACYGTTAC AGGTGTTGAA ATGTTCC...AAATGGT AATGCCTGGT GATAACGTTG	132
Enterococcus faecium	CAACAGTTAC TGGTGTTGAA ATGTTCC...AAATGGT CATGCCCGGT GACAACGT..	133
Escherichia coli	CTACCTGTAC TGGCGTTGAA ATGTTCC...AGATGGT AATGCCGGGC GACAACATCA	154
Fibrobacter succinogenes	ACGTCATCAC CGGTGTTGAA ATGTTCC...AAATGGT TACTCCGGGT GACACGGTCA	155
Flavobacterium ferrugineum	CTACCGTTAC AGGTGTTGAG ATGTTCC...AAATGGT TATGCCTGGT GATAACACCA	156
Gardnerella vaginalis	CCACCGTCAC CTCTATCGAG ACCTTCC...AAATGGT TCAGCCAGGC GATCACGCAA	135
Haemophilus influenzae	CTACTGTAAC GGGTGTTGAA ATGTTCC...AAATGGT AATGCCAGGC GATAACATCA	157
Helicobacter pylori	CGACTGTAAC CGGTGTAGAA ATGTTTA...AAATGGT TATGCCTGGC GATAATGTGA	158
Listeria monocytogenes	TAGTAGTAAC TGGAGTAGAA ATGTTCC...AAATGGT AAYGCCTGGT GATAACATTG	138
Micrococcus luteus	CCACTGTCAC CGGCATCGAG ATGTTCC...AGATGGT CATGCCCGGC GACAACACCG	159
Mycobacterium tuberculosis	CCACCGTCAC CGGTGTGGAG ATGTTCC...AGATGGT GATGCCCGGT GACAACACCA	160

Mycoplasma genitalium	CAGTTGTTAC TGGAATTGAA ATGTTCA...AAATGGT TCTACCTGGT GATAATGCTT	161
Neisseria gonorrhoeae	CCACCTGTAC CGGCGTTGAA ATGTTCC...AAATGGT AATGCCGGGT GAGAACGTAA	162
Rickettsia prowazekii	CGACTTGTAC AGGTGTAGAA ATGTTCA...AGATGGT TATGCCTGGA GATAATGCTA	163
Salmonella typhimurium	CTACCTGTAC TGGCGTTGAA ATGTTCC...AGATGGT AATGCCGGGC GACAACATCA	164
Shewanella putida	CAACGTGTAC TGGTGTAGAA ATGTTCC...AGATGGT AATGCCAGGC GATAACATCA	165
Stigmatella aurantiaca	CGGTCATCAC GGGGGTGGAG ATGTTCC...AGATGGT GATGCCGGGA GACAACATCG	166
Staphylococcus aureus	CAACTGTTAC AGGTGTTGAA ATGTTCC...AAATGGT AATGCCTGGT GATAACGTTG	140
Staphylococcus epidermidis	CAACTGTTAC TGGTGTAGAA ATGTTCC...AAATGGT TATGCCTGGC GACAACGTTG	141
Streptococcus agalactiae	CAGTTGTTAC TGGTGTTGAA ATGTTCC...AAATGGT TATGCCTGGT GATAACGTTA	144
Streptococcus pneumoniae	CAGTTGTTAC TGGTGTTGAA ATGTTCC...AAATGGT AATGCCTGGT GATAACGTGA	145
Streptococcus pyogenes	CTGTTGTTAC TGGTGTTGAA ATGTTCC...AAATGGT TATGCCTGGT GATAACGTGA	167
Thiobacillus cuprinus	CCACCTGCAC CGGCGTGGAA ATGTTCA...AAATGGT CATGCCCGGC GATAATGTGA	168
Treponema pallidum	CAGTGGTTAC TGGCATTGAG ATGTTTA...ACATGGT GAAGCCGGGG GATAACACCA	169

	Ureaplasma urealyticum	CTGTTGTTAC AGGAATTGAA ATGTTTA...ATTTGGT TATGCCAGGT GATGACGTTG	170
	Wolinella succinogenes	CAACCGTAAC TGGCGTTGAG ATGTTCC...AGATGGT TATGCCTGGT GACAACGTTA	171
5	*Candida albicans*	GTGTTACCAC TGAAGTCAAR TCCGTTG...AGRAATT GGAAGAAAAT CCAAAATTCG	120
	Schizo-saccharomyces pombe	GTGTCACTAC CGAAGTCAAG TCTGTTG...AGAAGAT TGAGGAGTCC CCTAAGTTTG	
	Human	TGACAGGCAT TGAGATGTTC CACAAGA...AGAAGGAGCTTGCCATG CCCGGGGAGG	
10	Selected[a] equences[a]	ACIKKIAC IGGIGTIGAR ATGTT ATGGT IATGCCIGGI GAIAAYRT	
	Selected universal	SEQ ID NO:23	SEQ ID NO: 24[b]
15	primer sequences[a]:	ACIKKIAC IGGIGTIGAR ATGTT	AYRTT ITCICCIGGC ATIACCAT

The sequence numbering refers to the *E. coli tuf* gene fragment. Underlined nucleotides are identical to the selected sequence or match that sequence.

20 [a] "I" stands for inosine which is a nucleotide analog that can bind to any of the four nucleotides A, C, G or T. "K", "R" and "Y" designate nucleotide positions which are degenerated. "K" stands for T or G; "R" stands for A or G; "Y" stands for C or T.

[b] This sequence is the reverse complement of the above *tuf* sequence.

Annex II: **Strategy for the selection from *tuf* sequences of the amplification primers specific for the genus *Enterococcus* (continues on pages 53 and 54).**

	314	348	401	435	SEQ ID NO
Bacillus subtilis	CGCGACACTG AAAAACCATT CATGATGCCA	GTTGA...	CGCGG ACAAGTTAAA GTCGGTGACG	AAGTTGAAAT	148
Bacteroides fragilis	CGCGATGTTG ATAAACCTTT CTTGATGCCG	GTAGA...	ACTGG TGTTATCCAT GTAGGTGATG	AAATCGAAAT	149
Burkholderia cepacia	CGTGCAGTTG ACGGCGCGTT CCTGATGCCG	GTGGA...	CGCGG CATCGTGAAG GTCGGCGAAG	AAATCGAAAT	152
Chlamydia trachomatis	AGAGAAATTG ACAAGCCTTT CTTAATGCCT	ATTGA...	CGTGG AATTGTTAAA GTTTCCGATA	AAGTTCAGTT	153
Corynebacterium diphteriae	CGTGAGACCG ACAAGCCATT CCTCATGCCT	ATCGA...	CGTGG CTCCCTGAAG GTCAACGAGG	ACGTCGAGAT	126
Enterococcus avium	CGTGATACTG ACAAACCATT CATGATGCCA	GTCGA...	CGTGG ACAAGTTCGC GTTGGTGACG	AAGTTGAAAT	131
Enterococcus faecalis	CGTGATACTG ACAAACCATT CATGATGCCA	GTCGA...	CGTGG TGAAGTTCGC GTTGGTGACG	AAGTTGAAAT	132
Enterococcus faecium	CGTGACAACG ACAAACCATT CATGATGCCA	GTTGA...	CGTGG ACAAGTTCGC GTTGGTGACG	AAGTTGAAGT	133
Enterococcus gallinarum	CGTGATACTG ACAAACCATT CATGATGCCA	GTCGA...	CGTGG ACAAGTTCGC GTTGGTGATG	AAGTAGAAAT	134
Escherichia coli	CGTGCGATTG ACAAGCCGTT CCTGCTGCCG	ATCGA...	CGCGG TATCATCAAA GTTGGTGAAG	AAGTTGAAAT	154

Gardnerella vaginalis	CACGATCTTG ACAAGCCATT CTTGATGCCA ATCGA...CGTGG TAAGCTCCCA ATCAACACCC CAGTTGAGAT	135
Haemophilus influenzae	CGTGCGATTG ACCAACCGTT CCTTCTTCCA ATCGA...CGAGG TATTATCCGT ACAGGTGATG AAGTAGAAAT	157
Helicobacter pylori	AGAGACACTG AAAAAACTTT CTTGATGCCG GTTGA...AGAGG CGTGGTGAAA GTAGGCGATG AAGTGGAAAT	158
Listeria monocytogenes	CGTGATACTG ACAAACCATT CATGATGCCA GTTGA...CGTGG ACAAGTTAAA GTTGGTGACG AAGTAGAAGT	138
Micrococcus luteus	CGCGACAAGG ACAAGCCGTT CCTGATGCCG ATCGA...CGCGG CACCCTGAAG ATCAACTCCG AGGTCGAGAT	159
Mycobacterium tuberculosis	CGCGAGACCG ACAAGCCGTT CCTGATGCCG GTCGA...CGCGG CGTGATCAAC GTGAACGAGG AAGTTGAGAT	160
Mycoplasma genitalium	CGTGAAGTAG ATAAACCTTT CTTATTAGCA ATTGA...AGAGG TGAACTCAAA GTAGGTCAAG AAGTTGAAAT	161
Neisseria gonorrhoeae	CGTGCCGTGG ACAAACCATT CCTGCTGCCT ATCGA...CGAGG TATCATCCAC GTTGGTGACG AGATTGAAAT	162
Salmonella typhimurium	CGTGCGATTG ACAAGCCGTT CCTGCTGCCG ATCGA...CGCGG TATCATCAAA GTGGGCGAAG AAGTTGAAAT	164
Shewanella putida	CGTGACATCG ATAAGCCGTT CCTACTGCCA ATCGA...CGTGG TATTGTACGC GTAGGCGACG AAGTTGAAAT	165
Staphylococcus aureus	CGTGATTCTG ACAAACCATT CATGATGCCA GTTGA...CGTGG TCAAATCAAA GTTGGTGAAG AAGTTGAAAT	140
Staphylococcus epidermidis	CGTGATTCTG ACAAACCATT CATGATGCCA GTTGA...CGTGG TCAAATCAAA GTWGGTGAAG AAGTTGAAAT	141
Staphylococcus saprophyticus	CGTGATTCTG ACAAACCATT CATGATGCCA GTTGA...CGTGG TCAAATCAAA GTCGGTGAAG AAATCGARAT	142

Streptococcus agalactiae	CGTGATACTG ACAAACCTTT ACTTCTTCCA GTTGA...CGTGG TACTGTTCGT GTCAACGACG AAGTTGAAAT	144
Streptococcus pneumoniae	CGTGACACTG ACAAACCATT GCTTCTTCCA GTCGA...CGTGG TATCGTTAAA GTCAACGACG AAATCGAAAT	145
Streptococcus pyogenes	CGCGACACTG ACAAACCATT GCTTCTTCCA GTCGA...CGTGG TACTGTTCGT GTCAACGACG AAATCGAAAT	167
Ureaplasma urealyticum	CGTAGTACTG ACAAACCATT CTTATTAGCA ATTGA...CGTGG TGTATTAAAA GTTAATGATG AGGTTGAAAT	170
Selected sequences	TACTG ACAAACCATT CATGATG	GTTCGC GTTGGTGACG AAGTT

Selected genus-specific primer sequences:	SEQ ID NO: 13 TACTG ACAAACCATT CATGATG	SEQ ID NO: 14[a] AACTTC GTCACCAACG CGAAC

The sequence numbering refers to the *E. faecalis tuf* gene fragment. Underlined nucleotides are identical to the selected sequence or match that sequence.

[a] This sequence is the reverse complement of the above *tuf* sequence.

NOTE: The above primers also amplify *tuf* sequences from *Abiotrophia* species; this genus has recently been related to the *Enterococcus* genus by 16S rRNA analysis.

Annex III: **Strategy for the selection from *tuf* sequences of the amplification primers specific for the genus *Staphylococcus* (continues on pages 56 and 57).**

	385	420.....579	611	SEQ ID NO
Bacillus subtilis	TGGCCGTGTA GAACGCGGAC AAGTTAAAGT	CGG.....TTG CTAAACCAGG	TACAATCACT CCACACAGCA	148
Bacteroides fragilis	AGGTCGTATC GAAACTGGTG TTATCCATGT	AGG.....TTT GTAAACCGGG	TCAGATTAAA CCTCACTCTA	149
Burkholderia cepacia	GGGTCGTGTC GAGCGCGGCA TCGTGAAGGT	CGG.....TGG CGAAGCCGGG	TTCGATCACG CCGCACACGC	152
Chlamydia trachomatis	TGGACGTATT GAGCGTGGAA TTGTTAAAGT	TTC.....TTT GCTTGCCAAA	CAGTGTTAAA CCTCATACAC	153
Corynebacterium diphteriae	CGGCCGTGTT GAGCGTGGCT CCCTGAAGGT	CAA.....TTG TTAAGCCAGG	CGCTTACACC CCTCACACCG	126
Enterococcus faecalis	AGGACGTGTT GAACGTGGTG AAGTTCGCGT	TGG.....TAG CTAAACCAGC	TACAATCACT CCACACACAA	132
Enterococcus faecium	AGGTCGTGTT GAACGTGGAC AAGTTCGCGT	TGG.....TAG CTAAACCAGG	TACAATCACA CCTCRTACAA	133
Escherichia coli	CGGTCGTGTA GAACGCGGTA TCATCAAAGT	TGG.....TGG CTAAGCCGGG	CACCATCAAG CCGCACACCA	154
Gardnerella vaginalis	CGGTCGTGTT GAGCGTGGTA AGCTCCCAAT	CAA.....TGG CTGCTCCAGG	TTCTGTGACT CCACACACCA	135

Haemophilus influenzae	AGGTCGTGTA GAACGAGGTA TTATCCGTAC AGG.....TAG CGAAACCAGG TTCAATCACA CCACACACTG	157
Helicobacter pylori	AGGTAGGATT GAAAGAGGCG TGGTGAAAGT AGG.....TAT GCAAACCAGG TTCTATCACT CCGCACAAGA	158
Listeria monocytogenes	TGGACGTGTT GAACGTGGAC AAGTTAAAGT TGG.....TAG CTAAACCAGG TTCGATTACT CCACACACTA	138
Micrococcus luteus	CGGTCGCGCC GAGCGCGGCA CCCTGAAGAT CAA.....TGG TGGAGCCGGG CTCCATCACC CCGCACACCA	159
Mycobacterium tuberculosis	CGGACGTGTG GAGCGCGGCG TGATCAACGT GAA.....TCA CCAAGCCCGG CACCACCACG CCGCACACCG	160
Mycoplasma genitalium	AGGAAGAGTT GAAAGAGGTG AACTCAAAGT AGG.....TAG CAAAACCAGG CTCTATTAAA CCGCACAAGA	161
Neisseria gonorrhoeae	CGGCCGTGTA GAGCGAGGTA TCATCCACGT TGG.....TGG CCAAACGGGG TACTATCACT CCTCACACCA	162
Salmonella typhimurium	CGGTCGTGTA GAGCGCGGTA TCATCAAAGT GGG.....TGG CTAAGCCGGG CACCATCAAG CCGCACACCA	164
Shewanella putida	AGGTCGTGTT GAGCGTGGTA TTGTACGCGT AGG.....TAG CGAAGCCAGG TTCAATCAAC CCACACACTA	165
Staphylococcus aureus	AGGCCGTGTT GAACGTGGTC AAATCAAAGT TGG.....TAG CTGCTCCTGG TTCAATTACA CCACATACTG	140
Staphylococcus epidermidis	AGGCCGTGTT GAACGTGGTC AAATCAAAGT WGG.....TAG CTGCTCCTGG TTCTATTACA CCACACACAA	141
Staphylococcus saprophyticus	AGGCCGTGTT GAACGTGGTC AAATCAAAGT CGG.....TAG CTGCTCCTGG TACTATCACA CCACATACAA	142
Staphylococcus simulans	AGGCCGTGTT GAACGTGGTC AAATCAAAGT CGG.....TAG CAGCTCCTGG CTCTATTACT CCACACACAA	143

Streptococcus agalactiae	AGGACGTATC GACCGTGGTA CTGTTCGTGT CAA.....TTG CTAAACCAGG TTCAATCAAC CCACACACTA	144
Streptococcus pneumoniae	AGGACGTATC GACCGTGGTA TCGTTAAAGT CAA.....TCG CTAAACCAGG TTCAATCAAC CCACACACTA	145
Ureaplasma urealyticum	TGGACGTGTT GAACGTGGTG TATTAAAAGT TAA.....TTG TAAAACCAGG ATCAATTAAA CCTCACCGTA	170
Selected sequences[a]	CCGTGTT GAACGTGGTC AAATCAAA GCTCCTGG YWCWATYACA CCACAYA	
Selected genus-specific primer sequences[a]:	SEQ ID NO: 17 CCGTGTT GAACGTGGTC AAATCAAA	SEQ ID NO: 18[b] TRTGTGGT GTRATWGWRC CAGGAGC

The sequence numbering refers to the *S.aureus tuf* gene fragment. Underlined nucleotides are identical to the selected sequence or match that sequence.

[a] "R", "W" and "Y" designate nucleotide positions which are degenerated. "R" stands for A or G; "W", for A or T; "Y", for C or T.

[b] This sequence is the reverse complement of the above *tuf* sequence.

Annex IV: **Strategy for the selection from *tuf* sequences of the amplification primers specific for the species *Candida albicans*** **(continues on pages 59 and 60).**

	58	90	181	213	SEQ ID NO
Candida albicans	CGTCAAGAAG GTTGGTTACA ACCCAAAGAC	TGT...	CAA ATCCGGTAAA GTTACTGGTA AGACCTTGTT		120
Candida glabrata	CATCAAGAAG GTCGGTTACA ACCCAAAGAC	TGT...	CAA GGCTGGTGTC GTCAAGGGTA AGAYCTTGTT		121
Candida krusei	CATCAAGAAG GTTGGTTACA ACCCAAAGAC	TGT...	CAA GGCAGGTGTT GTTAAGGGTA AGACCTTATT		122
Candida parapsilosis	CGTCAAGAAG GTTGGTTACA ACCCTAAAGC	TGT...	TAA AGCTGGTAAG GTTACCGGTA AGACCTTGTT		123
Candida tropicalis	CGTCAAGAAG GTTGGTTACA ACCCTAAGGC	TGT...	CAA GGCTGGTAAG GTTACCGGTA AGACTTTGTT		124
Schizo-saccharomyces pombe	CATCAAGAAG GTCGGTTTCA ACCCCAAGAC	CGT...	CAA GGCTGGTGTC GTCAAGGGTA AGACTCTTTT		
Human	GGAGATCCGG GAGCTGCTCA CCGAGTTTGG	CTA...	GTT AGGCCTGAAG TCTGTGCAGA AGCTACTGGA		
Chlamydia trachomatis	GGAGCTGCGC GAGCTGCTCA GCAAGTACGG	CTT...	CAA ATG..... ..TATTCTGG AGCTGATGAA		153
Corynebacterium diphteriae	GGAGATCCRT GAGCTGCTCG CTGAGCAGGA	TTA...	GAA GTGGACCCAG TCCATCATCG ACCTCATGCA		126
Enterococcus faecalis	GGAAGTTCGT GACTTATTAT CAGAATACGA	TTT.....	...TGAAGAA AAAATCTTAG AATTAATGGC		132
Escherichia coli	GGAAGTTCGT GAACTTCTGT CTCAGTACGA	CTT.....	..GGGAAGCG AAAATCCTGG AACTGGCTGG		154

	Flavobacterium ferrugineum	CGAGGTTCGC GAAGAACTGA CTAAACGCGG TTT..... ..GGGTTAAA GAAATTGAAA ACCTGATGGA	156
	Gardnerella vaginalis	AGAGGTCCGT GACCTCCTCG AAGAAAACGG CTT...CAA GTGGGTAGAG ACCGTCAAGG AACTCATGAA	135
5	*Haemophilus influenzae*	GGAAGTTCGT GAACTTCTAT CTCAATATGA CTT..... ..GGGAAGAA AAAATCCTTG AGTTAGCAAA	157
	Listeria monocytogenes	GGAAATTCGT GATCTATTAA CTGAATATGA ATT..... ..GGGAAGCT AAAATTGACG AGTTAATGGA	138
	Micrococcus luteus	GGAAGTCCGT GAGTTGCTGG CTGCCCAGGA ATT...CAA GTGGGTCGAG TCTGTCACAC AGTTGATGGA	159
10	*Neisseria gonorrhoeae*	GGAAATCCGC GACCTGCTGT CCAGCTACGA CTT..... ..ACGAAGAA AAAATCTTCG AACTGGCTAC	162
	Salmonella typhimurium	GGAAGTTCGC GAACTGCTGT CTCAGTACGA CTT..... ..GGGAAGCG AAAATCATCG AACTGGCTGG	164
15	*Staphylococcus aureus*	GGAAGTTCGT GACTTATTAA GCGAATATGA CTT..... ...CGAAGAA AAAATCTTAG AATTAATGGA	140
	Streptococcus pneumoniae	GGAAATCCGT GACCTATTGT CAGAATACGA CTT..... ...CGAAGAC ATCGTTATGG AATTGATGAA	145
	Treponema pallidum	AGAGGTGCGT GATGCGCTTG CTGGATATGG GTT...GGA GGATGCAGCT TGTATTGAGG AACTGCTTGC	169
20			

Selected sequences	CAAGAAG GTTGGTTACA ACCCAAAGA	ATCCGGTAAA GTTACTGGTA AGACCT
Selected species-specific primer sequences:	SEQ ID NO: 11 CAAGAAG GTTGGTTACA ACCCAAAGA	SEQ ID NO: 12[a] AGGTCTTACC AGTAACTTTAC CGGAT

The sequence numbering refers to the *Candida albicans tuf* gene fragment. Underlined nucleotides are identical to the selected sequence or match that sequence.

[a] This sequence is the reverse-complement of the above *tuf* sequence.

Annex V: **Strategy for the selection from the *recA* gene of the amplification primers specific for the genus *Streptococcus* (continues on pages 62 and 63).**

	415	449...540	574 SEQ ID NO
Bordetella pertussis	CTCGAGATCA CCGACGCGCT GGTGCGCTCG GGCTC...GGCCC GCCTGATGAG CCAGGCGCTG CGCAAGCTGA		
Burkholderia cepacia	CTCGAAATCA CCGATGCGCT GGTGCGCTCG GGCTC...GGCCC GCCTGATGTC GCAGGCGCTG CGCAAGCTGA		
Campylobacter jejuni	TTAGAAATTG TAGAAACTAT AGCAAGAAGT GGCGC...AGCAA GACTTATGTC TCAAGCTCTA AGAAAACTTA		
Chlamydia trachomatis	TTGAGTATTG CAGAGCTCTT AGCGCGTTCT GGAGC...AGCTC GCATGATGTC GCAGGCTCTA CGCAAATTAA		
Clostridium perfringens	TTAGAAATAA CAGAAGCTTT AGTTAGATCA GGAGC...AGCTA GATTAATGTC ACAAGCCTTA AGAAAGTTAA		
Corynebacterium pseudotuberculosis	CTGGAGATTG CAGATATGCT TGTTCGCTCT GGAGC...AGCGC GTTTGATGAG TCAGGCGCTG CGTAAGATGA		
Enterobacter agglomerans	CTGGAAATCT GTGATGCGCT GACCCGTTCA GGCGC...AGCTC GTATGATGAG CCAGGCGATG CGTAAGCTTG		
Enterococcus faecium	TTAGAGATTG CCGATGCCTT AGTTTCAAGT GGTGC...AGCTC GACTAATGTC TCAAGCACTA CGTAAATTAT		
Escherichia coli	CTGGAAATCT GTGACGCCCT GGCGCGTTCT GGCGC...GGCAC GTATGATGAG CCAGGCGATG CGTAAGCTGG		

Haemophilus influenzae	GCGAACAGAA GAATAGAATT TTAATGCATT ACCGC...GACCT GTGAGTTTAC GCAAAGCTTG AGACATTAAA	
Helicobacter pylori	TTAGAAATTT TAGAAACGAT CACCAGAAGC GGAGG...AGCAA GGCTTATGAG CCATGCGTTA AGAAAAATCA	
Lactococcus lactis	CTTCAAATTG CTGAAAAATT GATTACTTCT GGAGC...AGCAC GTATGATGTC ACAAGCCATG CGTAAACTTG	
Legionella pneumophila	CTGGAAATTA CTGATATGCT GGTGCGTTCT GCAGC...GGCAA GATTGATGTC GCAAGCCCTG CGTAAATTGA	
Mycoplasma genitalium	TTTGCTCTTA TCGAATCATT AATTAAAACA AACAA...TGCAA GAATGATGTC AAAAGGTTTG CGAAGAATAC	
Neisseria gonorrhoeae	TTGGAAATCT GCGACACGCT CGTCCGTTCG GGCGG...GGCGC GCCTGATGAG TCAGGCTTTG CGCAAACTGA	
Proteus mirabilis	CTGGAAATTT GTGATGCATT ATCTCGCTCT GGTGC...CGCAC GTATGATGAG CCAAGCTATG CGTAAACTAG	
Pseudomonas aeruginosa	CTGGAAATCA CCGACATGCT GGTGCGCTCC AACGC...GGCAC GCCTGATGTC CCAGGCGCTG CGCAAGATCA	
Serratia marcescens	CTGGAAATCT GTGATGCGCT GACCCGCTCC GGCGC...GGCGC GCATGATGAG CCAGGCGATG CGTAAGCTGG	
Shigella flexneri	CTGGAAATCT GTGACGCCCT GGCGCGTTCT GGCGC...GGCAC GTATGATGAG CCAGGCGATG CGTAAGCTGG	
Staphylococcus aureus	CTTGAAATCG CCGAAGCATT TGTTAGAAGT GGTGC...AGCTC GTTTAATGTC ACAAGCGTTA CGTAAACTTT	
Streptococcus gordonii	TTAGAAATTG CAGGAAAATT GATTGACTCT GGGGC.......	32
Streptococcus mutans	CTTGAAATTG CAGGGAAATT GATTGATTCT GGCGC...AGCAC GCATGATGAG TCAAGCGATG CGTAAATTAT	33

Streptococcus pneumoniae	CTTGAGATTG CGGGAAAATT GATTGACTCA GGTGC...GGCTC GTATGATGAG CCAGGCCATG CGTAAACTTG	34
Streptococcus pyogenes	CTTGAAATTG CAGGTAAATT GATTGATTCT GGTGC...AGCAC GTATGATGAG TCAGGCCATG CGTAAATTAT	35
Streptococcus salivarius	CTCGAAATTG CAGGTAAGCT GATTGACTCT GGTGC...AGCGC GTATGATGAG TCAAGCCATG CGTAAACTTT	36
Vibrio cholerae	CTGGAAATTT GTGATGCACT GGCTCGCTCT GGTGC...AGCGC GTATGTTGTC GCAAGCAATG CGTAAACTGA	
Yersinia pestis	CTGGAAATTT GTGATGCGCT GACTCGCTCT GGTGC...CGCGC GTATGATGAG CCAGGCTATG CGTAAGCTGG	
Selected sequences[a]	GAAATTG CAGGIAAATT GATTGA	ATGATGAG TCAIGCCATG CGTAA
Selected genus-specific primer sequences[a]:	SEQ ID NO: 21 GAAATTG CAGGIAAATT GATTGA	SEQ ID NO: 22[b] TTACGCAT GGCITGACTC ATCAT

The sequence numbering refers to the *S.pneumoniae recA* sequence. Underlined nucleotides are identical to the selected sequence or match that sequence.

[a] "I" stands for inosine which is a nucleotide analog that can bind to any of the four nucleotides A, C, G or T.

[b] This sequence is the reverse complement of the above *recA* sequence.

Annex VI: Specific and ubiquitous primers for DNA amplification

SEQ ID NO	Nucleotide sequence	Originating DNA fragment	
		SEQ ID NO	Nucleotide position
Bacterial species: ***Enterococcus faecium***			
1	5'-TGC TTT AGC AAC AGC CTA TCA G	26[a]	273-294
2[b]	5'-TAA ACT TCT TCC GGC ACT TCG	26[a]	468-488
Bacterial species: ***Listeria monocytogenes***			
3	5'-TGC GGC TAT AAA TGA AGA GGC	27[a]	339-359
4[b]	5'-ATC CGA TGA TGC TAT GGC TTT	27[a]	448-468
Bacterial species: ***Neisseria meningitidis***			
5	5'-CCA GCG GTA TTG TTT GGT GGT	28[a]	56-76
6[b]	5'-CAG GCG GCC TTT AAT AAT TTC	28[a]	212-232
Bacterial species: ***Staphylococcus saprophyticus***			
7	5'- AGA TCG AAT TCC ACA TGA AGG TTA TTA TGA	29[c]	290-319
8[b]	5'- TCG CTT CTC CCT CAA CAA TCA AAC TAT CCT	29[c]	409-438
Bacterial species: ***Streptococcus agalactiae***			
9	5'-TTT CAC CAG CTG TAT TAG AAG TA	30[a]	59-81
10[b]	5'-GTT CCC TGA ACA TTA TCT TTG AT	30[a]	190-212
Fungal species: ***Candida albicans***			
11	5'-CAA GAA GGT TGG TTA CAA CCC AAA GA	120[c]	61-86
12[b]	5'-AGG TCT TAC CAG TAA CTT TAC CGG AT	120[c]	184-209

[a] Sequences from databases.

[b] These sequences are from the opposite DNA strand of the sequence of the originating fragment given in the Sequence Listing.

[c] Sequences determined by our group.

Annex VI: Specific and ubiquitous primers for DNA amplification **(continues on next page)**

SEQ ID NO	Nucleotide sequence	Originating DNA fragment	
		SEQ ID NO	Nucleotide position
Bacterial genus: ***Enterococcus***			
13	5'-TAC TGA CAA ACC ATT CAT GAT G	131-134[a,b]	319-340[c]
14[d]	5'-AAC TTC GTC ACC AAC GCG AAC	131-134[a,b]	410-430[c]
Bacterial genus: ***Neisseria***			
15	5'-CTG GCG CGG TAT GGT CGG TT	31[e]	21-40[f]
16[d]	5'-GCC GAC GTT GGA AGT GGT AAA G	31[e]	102-123[f]
Bacterial genus: ***Staphylococcus***			
17	5'-CCG TGT TGA ACG TGG TCA AAT CAA A	140-143[a,b]	391-415[g]
18[d]	5'-TRT GTG GTG TRA TWG WRC CAG GAG C	140-143[a,b]	584-608[g]
19	5'-ACA ACG TGG WCA AGT WTT AGC WGC T	140-143[a,b]	562-583[g]
20[d]	5'-ACC ATT TCW GTA CCT TCT GGT AAG T	140-143[a,b]	729-753[g]
Bacterial genus: ***Streptococcus***			
21	5'-GAA ATT GCA GGI AAA TTG ATT GA	32-36[e]	418-440[h]
22[d]	5'-TTA CGC ATG GCI TGA CTC ATC AT	32-36[e]	547-569[h]
Universal primers			
23	5'-ACI KKI ACI GGI GTI GAR ARG TT	118-146[a,b] 147-171[a,e]	493-515[i]
24[d]	5'-AYR TTI TCI CCI GGC ATI ACC AT	118-146[a,b] 147-171[a,e]	778-800[i]

[a] These sequences were aligned to derive the corresponding primer.

[b] *tuf* sequences determined by our group.

[c] The nucleotide positions refer to the *E. faecalis tuf* gene fragment (SEQ ID NO: 132).

[d] These sequences are from the opposite DNA strand of the sequence of the originating fragment given in the Sequence Listing.

[e] Sequences from databases.

[f] The nucleotide positions refer to the *N. meningitidis asd* gene fragment (SEQ ID NO: 31).

[g] The nucleotide positions refer to the *S. aureus tuf* gene fragment (SEQ ID NO: 140).

[h] The nucleotide positions refer to the *S. pneumoniae recA* gene (SEQ ID NO: 34).

[i] The nucleotide positions refer to the *E. coli tuf* gene fragment (SEQ ID NO: 154).

Annex VI: Specific and ubiquitous primers for DNA amplification

SEQ ID NO	Nucleotide sequence	Originating DNA fragment	
		SEQ ID NO	Nucleotide position
Antibiotic resistance gene: *bla*$_{tem}$			
37	5'-CTA TGT GGC GCG GTA TTA TC	-	-
38	5'-CGC AGT GTT ATC ACT CAT GG	-	-
39	5'-CTG AAT GAA GCC ATA CCA AA	-	-
40	5'-ATC AGC AAT AAA CCA GCC AG	-	-
Antibiotic resistance gene: *bla*$_{shv}$			
41	5'-TTA CCA TGA GCG ATA ACA GC	-	-
42	5'-CTC ATT CAG TTC CGT TTC CC	-	-
43	5'-CAG CTG CTG CAG TGG ATG GT	-	-
44	5'-CGC TCT GCT TTG TTA TTC GG	-	-
Antibiotic resistance gene: *bla*$_{rob}$			
45	5'-TAC GCC AAC ATC GTG GAA AG	-	-
46	5'-TTG AAT TTG GCT TCT TCG GT	-	-
47	5'-GGG ATA CAG AAA CGG GAC AT	-	-
48	5'-TAA ATC TTT TTC AGG CAG CG	-	-
Antibiotic resistance gene: *bla*$_{oxa}$			
49	5'-GAT GGT TTG AAG GGT TTA TTA TAA G	110[a]	686-710
50[b]	5'-AAT TTA GTG TGT TTA GAA TGG TGA T	110[a]	802-826
Antibiotic resistance gene: *blaZ*			
51	5'-ACT TCA ACA CCT GCT GCT TTC	111[a]	511-531
52[b]	5'-TGA CCA CTT TTA TCA GCA ACC	111[a]	663-683
Antibiotic resistance gene: *aadB*			
53	5'-GGC AAT AGT TGA AAT GCT CG	-	-
54	5'-CAG CTG TTA CAA CGG ACT GG	-	-
Antibiotic resistance gene: *aacC1*			
55	5'-TCT ATG ATC TCG CAG TCT CC	-	-
56	5'-ATC GTC ACC GTA ATC TGC TT	-	-

[a] Sequences from databases.

[b] These sequences are from the opposite DNA strand of the sequence of the originating fragment given in the Sequence Listing.

Annex VI: Specific and ubiquitous primers for DNA amplification

SEQ ID NO	Nucleotide sequence	Originating DNA fragment	
		SEQ ID NO	Nucleotide position
Antibiotic resistance gene: ***aacC2***			
57	5'-CAT TCT CGA TTG CTT TGC TA	-	-
58	5'-CCG AAA TGC TTC TCA AGA TA	-	-
Antibiotic resistance gene: ***aacC3***			
59	5'-CTG GAT TAT GGC TAC GGA GT	-	-
60	5'-AGC AGT GTG ATG GTA TCC AG	-	-
Antibiotic resistance gene: ***aac6'-IIa***			
61	5'-GAC TCT TGA TGA AGT GCT GG	112[a]	123-142
62[b]	5'-CTG GTC TAT TCC TCG CAC TC	112[a]	284-303
63	5'-TAT GAG AAG GCA GGA TTC GT	112[a]	445-464
64[b]	5'-GCT TTC TCT CGA AGG CTT GT	112[a]	522-541
Antibiotic resistance gene: ***aacA4***			
65	5'-GAG TTG CTG TTC AAT GAT CC	-	-
66	5'-GTG TTT GAA CCA TGT ACA CG	-	-
Antibiotic resistance gene: ***aad(6')***			
173	5'-TCT TTA GCA GAA CAG GAT GAA	-	-
174	5'-GAA TAA TTC ATA TCC TCC G	-	-
Antibiotic resistance gene: ***vanA***			
67	5'-TGT AGA GGT CTA GCC CGT GT	-	-
68	5'-ACG GGG ATA ACG ACT GTA TG	-	-
69	5'-ATA AAG ATG ATA GGC CGG TG	-	-
70	5'-TGC TGT CAT ATT GTC TTG CC	-	-
Antibiotic resistance gene: ***vanB***			
71	5'-ATT ATC TTC GGC GGT TGC TC	116[a]	22-41
72[b]	5'-GAC TAT CGG CTT CCC ATT CC	116[a]	171-190
73	5'-CGA TAG AAG CAG CAG GAC AA	116[a]	575-594
74[b]	5'-CTG ATG GAT GCG GAA GAT AC	116[a]	713-732

[a] Sequences from databases.

[b] These sequences are from the opposite DNA strand of the sequence of the originating fragment given in the Sequence Listing.

Annex VI: Specific and ubiquitous primers for DNA amplification

SEQ ID NO	Nucleotide sequence	Originating DNA fragment	
		SEQ ID NO	Nucleotide position
Antibiotic resistance gene: ***vanC***			
75	5'-GCC TTA TGT ATG AAC AAA TGG	117[a]	373-393
76[b]	5'-GTG ACT TTW GTG ATC CCT TTT GA	117[a]	541-563
Antibiotic resistance gene: ***msrA***			
77	5'-TCC AAT CAT TGC ACA AAA TC	-	-
78	5'-AAT TCC CTC TAT TTG GTG GT	-	-
79	5'-TCC CAA GCC AGT AAA GCT AA	-	-
80	5'-TGG TTT TTC AAC TTC TTC CA	-	-
Antibiotic resistance gene: ***satA***			
81	5'-TCA TAG AAT GGA TGG CTC AA	-	-
82	5'-AGC TAC TAT TGC ACC ATC CC	-	-
Antibiotic resistance gene: ***aac(6')-aph(2")***			
83	5'-CAA TAA GGG CAT ACC AAA AAT C	-	-
84	5'-CCT TAA CAT TTG TGG CAT TAT C	-	-
85	5'-TTG GGA AGA TGA AGT TTT TAG A	-	-
86	5'-CCT TTA CTC CAA TAA TTT GGC T	-	-
Antibiotic resistance gene: ***vat***			
87	5'-TTT CAT CTA TTC AGG ATG GG	-	-
88	5'-GGA GCA ACA TTC TTT GTG AC	-	-
Antibiotic resistance gene: ***vga***			
89	5'-TGT GCC TGA AGA AGG TAT TG	-	-
90	5'-CGT GTT ACT TCA CCA CCA CT	-	-
Antibiotic resistance gene: ***ermA***			
91	5'-TAT CTT ATC GTT GAG AAG GGA TT	113[a]	370-392
92[b]	5'-CTA CAC TTG GCT TAG GAT GAA A	113[a]	487-508

[a] Sequences from databases.

[b] These sequences are from the opposite DNA strand of the sequence of the originating fragment given in the Sequence Listing.

Annex VI: Specific and ubiquitous primers for DNA amplification

SEQ ID NO	Nucleotide sequence	Originating DNA fragment	
		SEQ ID NO	Nucleotide position
Antibiotic resistance gene: ***ermB***			
93	5'-CTA TCT GAT TGT TGA AGA AGG ATT	114[a]	366-389
94[b]	5'-GTT TAC TCT TGG TTT AGG ATG AAA	114[a]	484-507
Antibiotic resistance gene: ***ermC***			
95	5'-CTT GTT GAT CAC GAT AAT TTC C	115[a]	214-235
96[b]	5'-ATC TTT TAG CAA ACC CGT ATT C	115[a]	382-403
Antibiotic resistance gene: ***mecA***			
97	5'-AAC AGG TGA ATT ATT AGC ACT TGT AAG	-	-
98	5'-ATT GCT GTT AAT ATT TTT TGA GTT GAA	-	-
Antibiotic resistance gene: ***int***			
99	5'-GTG ATC GAA ATC CAG ATC C	-	-
100	5'-ATC CTC GGT TTT CTG GAA G	-	-
101	5'-CTG GTC ATA CAT GTG ATG G	-	-
102	5'-GAT GTT ACC CGA GAG CTT G	-	-
Antibiotic resistance gene: ***sul***			
103	5'-TTA AGC GTG CAT AAT AAG CC	-	-
104	5'-TTG CGA TTA CTT CGC CAA CT	-	-
105	5'-TTT ACT AAG CTT GCC CCT TC	-	-
106	5'-AAA AGG CAG CAA TTA TGA GC	-	-

[a] Sequences from databases.

[b] These sequences are from the opposite DNA strand of the sequence of the originating fragment given in the Sequence Listing.